Gas Chromatographic Identification of Trace Amounts of Organic Compounds

by

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STATEMENT

Except as acknowledged herein, this thesis contains no material which has been accepted for the award of any other degree or diploma in any university, and that, to the best of my knowledge and belief, this thesis contains no copy or paraphrase of material previously published or written by another person, except when due reference is made in the text of the thesis.

ABSTRACT

Methods for the identification of gas chromatographic separated components of essential oils have been investigated.

The use of relative retention data was found to be of value for identification only when known standards are available for comparison. Tabulated Kovats indices, available from references, were found to be unreliable for the identification of unknown compounds. These techniques may be of value for preliminary identification of some compounds, however it was found that spectroscopic and chemical reaction data were necessary in order to postively identify unknown compounds.

Combined gas chromatography - mass spectrometry computer techniques enabled the rapid identification of the majority of compounds present in these complex mixtures. Compounds identified by this technique were found to be in agreement with many previously identified by retention data. There were limitations in the identification of compounds with similar spectra and the spectra of unknown compounds not being included in the data base.

Where other spectroscopic data was necessary for identification purposes, an off-line gas chromatographic sampling device was constructed which enabled the collection of nanogram amounts of compounds eluting from a gas chromatographic column. Compounds collected this way were able to have their Raman spectra recorded without any further sample manipulation. The technique was also used for the recording of infrared, and nuclear magnetic resonance spectra enabling positive identification of some compounds.

Techniques of reaction gas chromatography mass spectrometry were developed. Hydrogenation techniques were found useful for the

determination of the number of double bonds in a compound whilst carbon skeleton chromatography - mass spectromatry was used for the identification of the hydrocarbon skeleton of unidentified compounds. Subtraction gas chromatography enabled the tentative identification of the alcohol functional group in some compounds.

It was concluded that, due to the complex nature of the essential oils investigated, a single technique is not available that enables complete identification of all components. A combination of all these methods must often be used for the complete identification of components in essential oils.

ABBREVIATIONS

GC	-	Gas Chromatography
FID	-	Flame Ionization Detector
MS	-	Mass Spectrometer
TIC	-	Total Ion Current
HPLC	-	High Performance Liquid Chromatography
CW 20m	-	Carbowax 20m Stationary Phase
OV-101	-	OV Silicone Stationary Phase
SCOT	-	Support Coated Open Tubular (column)
WCOT	-	Wall Coated Open Tubular (column)
FTIR	-	Fourier Transform Infra Red (spectrophotometry)
RRT	-	Relative Retention Time
KI	-	Kovats Indices
LR	-	Laser Raman (spectroscopy)
NMR	-	Nuclear Magnetic Resonance (spectroscopy)
EI	-	Electron Impact
CI	-	Chemical Ionization
FI	-	Field Ionization
FD	-	Field Desorption
tr	-	trace
I	-	Kovats Indices
TPKI	-	Temperature Programmed Kovats Indices
TLC	-	Thin Layer Chromatography

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CHAPTER 1

1. General Introduction

The identification of components separated by gas chromatography (GC) is an important technique for structural assignment in organic analytical chemistry.

Since GC was first introduced as an analytical technique (1) many different identification systems based on a compound's retention characteristic have been proposed with varying degrees of success. The most widely used at present is the Kovats system (2) where a compound's elution is compared with an homologous series of n-alkanes.

Increased credence can be given to identification assigned on retention data by two dimensional chromatography, i.e. separation on one coloumn followed by separation on another column with a different phase. The resolving power of this GC system is thus increased and the possibility of compounds co-chromatographing is less. By utilization of a high resolution dual phase chromatographic system a considerable percentage of routine identifications may often be established with reasonable certainty. Retention criteria are also of importance where structurally related compounds yield similar spectra.

Chemical reaction techniques to separate or modify components prior to GC analysis may be carried out in an isolated system, e.g. a syringe or by a reaction chamber incorporated into the GC system. Information as to the functional group, position and number of double bonds and the carbon skeleton of the compound may be obtained.

Gas chromatography either coupled on-line or off-line with spectroscopic techniques such as mass spectrometry (MS), infrared spectroscopy (IR), laser-Raman spectroscopy (LR) and nuclear magnetic

resonance spectroscopy (NMR) must often be used, however, in order to obtain additional information enabling a higher degree of certainty for structural assignment.

In this research project it was proposed to investigate the use of GC retention measurements for the identification of components present in complex mixtures. Where these techniques were inadequate it was also intended to examine both on-line and off-line spectroscopic methods such as laser-Raman, IR, GC-mass spectrometry (GC-MS) and NMR, that may be necessary for correct structural identification.

It was also planned to study the usefulness of pre-column derivatization coupled with GC and GC-MS to enable the positive identification of closely related structures that may exhibit similar retention characteristics and spectroscopic properties.

The group of compounds chosen for this study was the terpene components of the leaf oils of Atherosperma moschatum (Sassafras, family Monimiaceae) and Leptospermum lanigerum (Woolly Tea-tree, family Myrtaceae). Sassafras is widespread in rain forests in Tasmania, Victoria, New South Wales and Queensland, and is used for flavouring beverages, timber and craftwork. Woolly Tea-tree occurs in damp places, river banks, montane grasslands and rainforests in Tasmania, Victoria, New South Wales, Queensland and South Australia.

As the essential oil industry is one of increasing significance in Tasmania, the refinement and development of these organic analytical methods should be of value for further investigation of similar essential oils.

Prior to this investigation it was necessary to review the recent relevant literature in this field which is summarized in the following sections.

CHAPTER 2

2. Literature Review

2.1 Gas Chromatographic Identification Techniques:

The measurements of a compound's retention in a GC column may yield information sufficient for the characterization of a compound or information complementary to other identification techniques.

Of the various retention data measurements, those compared relative to a single arbitrary standard are widely used for data presentation (1). The system introduced by Kovats (2) where a compound's retention is compared relative to a series of homologous compounds has gained acceptance because of its better reproducibility compared to other forms of retention data.

As the retention time or volume of a compound in a GC system is dependent on many factors it is important to consider those chemical and physical processes which may contribute to GC separation, compound retention, and accuracy and precision of measurement.

Unfortunately, some factors affecting the degree of accuracy and precision of inter- and intra-laboratory reproducibility (3,4) can not be controlled by the laboratory worker. These factors affecting measurement of retention data have been reviewed, e.g. Pacakova et al (5), Ettre (6) and Haken (7).

2.1.1 Retention Measurements Systems for Compound Identification

The direct comparison of retention times of eluting components for identification is dependent on the reproducibility of both column and analytical conditions. As these parameters are always changing, retention times or adjusted retention times are seldom used for identification.

Retention volume of a component is less dependent on analytical conditions but their use is not generally accepted as this calculation requires the knowledge of values which are not generally known or may change during the operating lifetime of the GC column.

The measurement of a compound's retention relative to a known standard may eliminate some of the variables associated with retention time and volume measurements. However, for reproducible results, identical conditions of column temperature, gas flow rate and accurate calculation of dead volume are necessary (7). Relative retention data for peak identification on a single column are limited and data calculated on two columns, of different polarity often provide for a more certain identification (7).

With certain homologous series, retention values can be related to either physical or structural properties of a compound in order to give additional identification criteria. James (1) found that data from each homologous series of compounds when compared with retention characteristics resulted in straight line relationships. The use of retention increment for sample characterization was proposed.

Differentiation between two isomeric compounds has been demonstrated by Tourres (8). It was found that retention data on liquid phases of two different polarities or at two different temperatures produce plots in which isomers give lines of parallel slope. Yabamuto et al (9) have recently used these techniques to identify many components in essential oils. They also demonstrated that these techniques eliminated the possibility of two or more components exhibiting the same retention behaviour in a GC column. This possibility is most likely to occur in low resolution packed columns and has been largely overcome by the use of high resolution capillary columns.

Measurement of the retention by Kovats indices where a compound's elution is compared with an homologous series of n-alkanes (2) has become a widely used technique for solute identification. Different methods of utilizing Kovats indices have been developed, direct table matching of either isothermal or temperature programmed data and retention index increment calculations.

Increment values, calculated using the relationships existing between temperature, retention index and molecular structure have been used for solute identification (10). These measurements can not usually be applied by themselves but have found use in conjunction with spectroscopic techniques.

Isothermal indices (2) are widely used as an identification technique and data for many classes of compounds have been published. Reproducibility and precision of measurement are higher for retention indices than for previously described methods, as standard compounds may be selected in such a way that their retention characteristics are similar to that of the measured compounds.

The disadvantage of using n-alkane standards with highly polar stationary phases has been investigated (17). Due to their complete non polarity causing low solubility in these phases, homologous series of compounds with polar functional groups have been proposed as standards for polar stationary phases.

Temperature programmed calculation of Indices has been reported (18). Van den Dool and Kratz replaced the logarithmic relationship that exists for isothermal conditions with an approximate linear relationship in order to utilize the advantages of temperature programmed GC. Giddings (19) showed that this approximation held true for higher members of a homologous series but departures from linearity were found for lower members of the series. The linearity of the relationship was found to be dependent on both temperature and the rate of temperature programming. Deviations from linearity in temperature programming in commercial instrumentation have been investigated. They were found to be more pronounced at either end of the program range and contributed to the non-linearity of the relationship (20).

To minimize these errors from the direct linear approximation of retention data, curve fitting approaches have been investigated and

reviewed (20). Of the techniques available, approximation by the use of a cubic spline function has been shown to have advantages over polygonal approximation due to smoothness of fit, and ease of calculation (21).

Interlaboratory reproducibility of retention Indices has been surveyed, however the results were found to be unsatisfactory (22). Schomburg and Dielman (23) have calculated that reproducibility should, under ideal conditions, be within one unit. Ettre (24) has proposed that the reliability of published data is sometimes much poorer than the possible reproductibility of such measurements.

2.1.2 Analytical Considerations for Retention Measurements

Where tabulated data are used for identification purposes, the precision with which these values can be determined and whether or not the measured values unambiguously characterize the given substance are the most important criteria. These factors affecting measurement of data have been reviewed (5, 22) and have been found to be dependent on instrumentation, the partition process, adsorption effects, the stationary phase, column efficiency and the calculation of gas hold up times.

Effects of the column on measurement of retention data have been investigated (24, 25, 26). The ideal column was considered to be one with high separation efficiency, high temperature stability, minimum tailing characteristics, and for components of low volatility, the column should contain small amounts of stationary phase with even film homogeneity.

Pacakova (5) calculated retention indices on packed and open tubular columns and concluded that the precision of measurement is substantially higher with open tubular columns and they should therefore be preferred for precise measurements of retention data.

Reproducibility of retention data on wall coated and support coated columns has been shown by Ettre (24) to be in close agreement. Data was

interchangeable for either type of column.

The use of capillary columns for retention measurements requires special sampling techniques in order to obtain reproducible results. Early methods of sample introduction (27) used a simple carrier gas inlet splitter which did not show discrimination with regard to concentration and volatility of smaple. These variations may result from incorrect gas flow geometry and temperature gradient effects. Novotny (28) found that although splitting injectors are adequate for the analysis of concentrated samples, problems can be encountered in trace analysis, particularly when the total amount of sample is limited.

Grob and Grob,(27, 29, 30) examined the direct injection of samples onto capillary columns. They demonstrated that with this technique, dilute solutions of less volatile trace components can be condensed at the column inlet with minimum band spreading whilst the more volatile solvent passes through the column without affecting the retention characteristics of the components. Direct injection techniques may also be used to concentrate trace sample components behind the condensed solvent band (30). This sample introduction technique ensures minimum peak broadening, and maintains column efficiency and accuracy of retention parameters.

Stationary phase composition and chemical inertness of the support and column material is of importance for precision in retention measurements. McReynolds (31) found that batch differences in stationary phases resulted in non reproducible data. Chemical reactions such as polymerization and catalytic decomposition (32) of the stationary phase may change its chemical nature and therefore its retention characteristics.

Precision of retention data has been investigated by Pacakova (5) who found that measurements could be affected by non uniformity of stationary phase film thickness. It was shown that the most pronounced deviations occurred for polar phases, where the retention index may

decrease with increased film thickness, due to absorption effects. For non polar compounds the retention index was found to increase with coating thickness due to increased solubility. A uniform film thickness must be considered necessary if reproducible retention measurements are to be made.

Effects of column materials on solute retention with identical stationary phases have been described (33). The degree of deactivation of the stationary phase support material was shown to have a marked effect on retention measurements, especially when using thin films of stationary phases. Grob and Grob (27) demonstrated that the active surface of the support material may protrude through the thin film layer and interact with polar compounds resulting in increased retention.

Accurate measurement of retention data requires that the column dead volume is well characterized. Methods utilized for calculation of this parameter are based on the linear relationship existing between the logarithm of the retention volume of an homologous series of n-alkanes and the carbon number (35, 36). Schomburg et al (26) noted that the use of lower members of homologous series should be avoided for this calculation as they may not show a linear relationship.

Retention parameters have been calculated without calculation of dead volume (37). Techniques are based on the third order relationship existing between the logarithm of differences of unadjusted retention times and carbon number. The major advantage of this method is that it offers the possibility of automated on-line calculation of retention indices by computer using unadjusted retention times.

Computer techniques (37, 38) for calculation of dead volume also have the advantage that retention information for a series of homologous compounds can provide a rapid statistical assessment of the operating parameters of a particular column.

The use of inert tracers for dead volume determination has been

discussed (22). It was found that they may be absorbed by the stationary support resulting in considerable errors in calculation of both relative retention and Kovats Indices. In practice it is advantageous to use a method based on carbon number calculations. Huber and Gerritze (22) have reviewed the errors resulting from the physical measurement of retention time. Errors of measurement of starting point were found to be due to the use of the time of injection instead of the time of entry of the sample into the column. Errors in the end point determination were due to the difference between elution time of the peak maxima, as indicated by the detector, and the true value. These differences can be minimized by increased resolution of successive peaks, faster detector response and improved sampling.

2.2 Ancillary Identification Techniques

2.2.1 Gas Chromatography - Mass Spectrometry

Of the spectroscopic techniques available, GC-MS is the most widely used for the analysis of complex mixtures. With the aid of computer techniques, identification of picogram amounts of gas chromatographic effluents by mass spectrometry is now an established method. The combination of these two techniques allows for the mass spectral analysis of a highly purified sample by either on-line or off-line techniques.

In the analysis of essential oils, GC-MS has advantages over other techniques in that reliable identification of individual components in complex mixtures may be undertaken in a short period of time. Some difficulties do exist (39) in that the spectra of similar compounds may not be readily distinguished, the direct comparison with literature spectra is not often applicable due to different ionization conditions, and the reference spectra of unknown samples may not be available.

Further limitations exist in the difficulty in distinguishing between stereoisomers and compounds that may rearrange via a common

intermediate, e.g. some monoterpenes. Mass spectrometry used in conjunction with other techniques such as GC retention measurements and chemical derivatization may help in solving these problems.

Coupling Gas Chromatography with Mass Spectrometry

Early studies of gas chromatography and mass spectrometry utilized the trapping of each component as it emerged from a GC column and the manual transfer to the batch inlet system of a mass spectrometer. Trapping and transfer of many components is, however, time consuming, and it is only applicable to low resolution packed columns. Compounds may also be chemically altered or contaminated during this process.

Direct interfacing (40) of GC and MS has overcome these problems and is accomplished by either effluent splitting or open split coupling.

Coupling by way of an effluent splitter allows only a few percent of the GC effluent into the MS (41). The efficiency of this system is low and no enrichment of the compounds in the carrier gas occurs. Gas chromatographic conditions and retention parameters are not affected and it is suitable for all types of columns.

Open split coupling may be accomplished by selecting columns for minimal carrier gas flow and connecting them directly to the ion source (42,43). Total efficiency is obtained in this technique by passing the total effluent into the MS. This is the most recommended technique (42,43,44), but is, however, restricted to small bore capillary columns of flow rates 1 - 5 mls/minute of helium. Resolution of the column and retention indices may also be affected by the vacuum at the column exit.

Analytical and instrumental conditions often require enrichment of a compound in the carrier gas. Rhyage (45) demonstrated that different rates of diffusion of different gases in an expanding jet stream could be used as an enrichment device in a GC-MS interface. Yields of 80 percent have been reported (45,46) with an enrichment factor of about

50. No adsorption effects have been noted and no influence on GC separation and retention properties occur.

The separator of Watson and Bieman (47) utilizes effusion through the pores of a sintered glass fritt for enrichment. A disadvantage of this system is that it is non adjustable and optimizes only within a certain flow range with yields of only 50 percent. GC separation properties have been investigated with this separator (48) and peak broadening, especially with capillary columns, has been noted.

Llewellyn and Littlejohn (49) have developed a separator using a dimethylsilicone membrane. Enrichment values of 10 to 20 with yields of 30 to 90 percent were obtained due to the permeability of the organic sample being many times that of the carrier gas. GC retention times were not found to be distorted since the system operates at atmospheric pressure and variable flow rates can be used. The major disadvantage of this enrichment process is that its operational temperature precludes some GC-MS determinations.

Mass Spectral Identification Techniques

Determination of the parent or molecular ion peak often provides sufficient information to establish the molecular formulae for molecules of low molecular weight.

The combination of unit resolution mass measurement of a molecular ion peak with that of a further peak corresponding to a molecular ion containing a heavy isotope in place of a common isotope may provide a means of reducing the number of molecular ion possibilities. When another isotopic peak can be included corresponding to a molecular ion containing several isotopes, a more definitive selection may be made for the parent molecular ion. This method has found value for the analysis of mixtures where fragment ion peaks are observed and only molecular ion peaks can be measured. Literature on this technique has been reviewed (50) regularly and the limiting factors of the method

have been noted (51). The technique may be of use in GC-MS where maximum peak intensities are produced by slow scanning over a limited mass range.

The reduction of the potential of the ionizing beam to near the appearance potential of the molecular ion reduces the intensity of the fragment peaks and increases the intensity of the molecular ion peak. By the selection of critical ionization voltages, molecular ion peaks may be produced that aid in the identification of particular groups of compounds in complex mixtures (51).

Peaks which are confused with the molecular ion peak may be often excluded on the basis of normal valency structure requirements. Selection of the molecular ion may be assisted by a structural requirement such as the "nitrogen rule" relating the number of atoms to the molecular weight of the molecules. A corollary of this (52) relates the presence of nitrogen atoms to the molecular weights of both the molecular ion and its fragments and thus aids in identification.

Fragmentation mechanism proposals may be used for identification if incomplete mass spectra are obtained from unstable and minor components eluted in a GC-MS system. With a recognizable molecular ion peak in an incomplete fragment ion pattern, the possible fragmentation mechanism may give information sufficient to determine functional groups and sometimes the carbon skeleton. Where conflicting data are found, especially with terpene spectra (53), it has been suggested that fragmentation mechanisms are investigated by isotope labelling techniques (54).

Information from studies of metastable ion peaks (50) may lead to the direct elucidation of the mechanisms of degradation reactions and hence sample identification. Metastable peaks may also be used for calculation of masses of the molecular ions of unstable compounds (56, 57). The technique has been reviewed (53, 58) and limitations

of both peak comparison and fragment mechanism from metastable transition methods in identification of terpenes have been noted (55).

The identification of a particular compound may be greatly assisted by the construction of the extracted ion current profile (61) of an intense ion from the spectrum of the compound. A peak will be observed in this profile at the position where the compound elutes from the gas chromatograph. If the ion is an anomalous fragment ion, the extracted ion current profile may contain almost as many peaks as the total ion current profile. The intense fragmentation observed in the EI spectra of terpenoids together with the similarity of many of their EI spectra renders this technique mostly unsuitable for fragment ions and insensitive for molecular ions. Chemical ionization spectra are better suited for the generation of extracted ion current profiles due to their simplicity.

Mass Spectral Ionization Techniques

Different ionization techniques have been developed for the production of ions in mass spectral identification including chemical ionization (CI), electron impact (EI), field ionization (FI) and field desorption (FD).

Electron impact techniques are the most widely used where a reasonable assumption can often be made as to the identity of the compound. Where difficulty in establishing the molecular weight of the parent ions occurs, other less energetic techniques may be required.

Chemical ionization - MS (CIMS) provides simpler fragmentation and is a complementary technique for the identification of complex structures. With CIMS, sample molecules are ionized by reaction with ionized or excited gas molecules, resulting in fragmentation that is relatively gentle and producing an abundance of high molecular weight particles.

Criteria for CIMS have been summarized recently by Hendricks and Bruins (59) who emphasized that under some conditions the technique fails to give molecular weight information for mono and sesquiterpene

alcohols using methane or isobutane as reagent gas.

Under negative chemical ionization conditions, molecules exhibit less fragmentation than in positive ion CIMS. The sensitivity of the ionization process is high for compounds containing electronegative elements and with the use of selective ion monitoring techniques, subpicogram detection limits are obtainable.

With hydroxyl ion-induced CIMS, Hendrick and Bruins (59) were able to produce abundant $(M-H)^-$ ions of most oxygenated mono and sesquiterpenes. They were able to characterize the compounds of interest in different essential oils of the same species by combined GC-OH^- negative ion CIMS. The extracted ion current profiles of the $(M-H)^-$ and $RCOO^-$ ions gave clear identification.

Adams et al (39) have suggested that CI techniques do not solve the problem of interpretation of similar spectra, as difficulties in obtaining reproducible mass ion intensities for a given compound exist. Other GC spectroscopic techniques are needed to solve this problem.

Computer Techniques

The improvement of combined GC-MS instrumentation has resulted in the routine generation of large amounts of data. This has necessitated a rapid development of computer-based methods for the interpretation of spectra, instrument control and special manipulative procedures.

Interpretation of mass spectral data is well suited to computer techniques due to the fact that except for a small percentage of peaks, e.g. metastable and multiply charged species, peaks occur at integral mass units. Basic computer techniques commonly used include library search, pattern recognition and interpretative techniques. All these methods are dependent on mass spectra data bases and libraries tabulated from collected data. An attempt has been made to identify all the existing collections of mass spectra (62), however these libraries can not be considered to be comprehensive.

An important consideration is the quality of the spectral data these libraries contain. Due to the fact that spectra are sensitive to various instrument parameters, a library may contain low quality spectra or spectra that are completely incorrect. Speck et al (63) have developed an algorithm to give a quality rating to mass spectra based on several parameters such as election voltage, peaks at M/z values above the molecular ion, isotopic abundance ratios, illogical neutral losses, total number of peaks related to the number of atoms, and the lowest M/z reported.

In using a similar algorithm it has been shown (64) that approximately 4% of the spectra in the NIH(EPA)MSDC data base received a quality index of zero. It was emphasised that the quality index is a good indicator that the spectrum is incorrect, however it is not as good an indicator that the spectrum is of high quality.

Several workers have investigated improving the quality of mass spectral libraries. Criteria for tuning a mass spectrometer using a standard compound with well defined peaks over a wide mass range have been investigated (65). Dillard et al (66) have published a set of standards for defining the class of a spectrum. The highest classification being for those spectra obtained under strict instrumental conditions and matching with in specificed limits a spectrum of the same compound on a second instrument.

Library search methods are widely used for identification of spectra where an index of similarity is defined from a library and the unknown. These include the Biemann search (67) and the PBM search of McLafferty et al (68). In the Biemann search, the library spectra are abbreviated by selecting the two most intense peaks in each fourteen mass units. The unknown spectrum is treated in the same manner and a similarity index is calculated between the two spectra. As this is a forward search technique it expects to find all peaks from the unknown spectrum in the reference

spectrum. Impurity peaks may therefore give a significant decrease in the similarity index.

The probability based matching (PBM) technique has been developed by McLafferty (68, 69). This is the most widely used reverse search technique and is based on a statistical analysis of a large library to determine the probability of any given peak in a spectrum chosen at random. This program has been tested with over 800 unknown mass spectra (70) and, for spectra of pure compounds, its performance was shown to be superior to the Biemann search.

Henneberg and co-workers (71, 72) have reported the development of a system differing from the Biemann and PBM method in the choice of peaks used in the search. The SICOM search selects certain characteristic ions which may be observed in the proximity of more intense but less characteristic fragments. It is applicable to the identification of components in mixtures and compounds of similar structure if the unknown compound in not in the library. It was considered superior to the PBM search for the retrieval of certain classes of compounds (72).

The self training interpretative and retrieval system (STIRS) of McLafferty and co-workers is the most extensive of the pattern recognition techniques (73). It consists of a number of nearest neighbour analyses based on characteristic ions, ion series and primary and secondary neutral losses in various mass ranges. Dayringer et al (74) have shown the ability of STIRS to detect a substructure and it has also been compared with other pattern recognition techniques and found to be superior (75). Dayringer and McLafferty (76) have used STIRS for the prediction of the number of rings plus double bonds in a compound, whilst Mun et al (77) have described the application of STIRS to predict the molecular weight of a compound. With the combination of these predictions they proposed predicting elemental compositions using STIRS.

Interpretative techniques have been developed (73, 78, 79) using

artificial intelligence methods to recognise mass spectral behaviour of particular classes of compounds. A program to generate all possible structures has been used by Masinter et al (80) where spectra are predicted from empirical mass spectrometry rules. Algorithms for calculation of predicted MS fragmentation from empirical data of known compounds have also been used (81). It is important to note that these interpretative methods are designed as an aid to, and not as a replacement for, human interpretation.

Identification by both GC and mass spectral data has been described by Blaisdell and Sweeley (82). By using a least squares method the best fit between the library spectrum in the retention index window and the chosen GC-MS scan is calculated. As retention indices are naturally ordered they give a further subsetting of the library for each unknown spectrum enabling a matching procedure with only a few of the spectra in the library. It has been found to be a faster, more definitive process as it refers only to a small set of definitive ions (83).

Previous attempts to identify terpenes by computer techniques have not been entirely satisfactory (39). By recording the spectra using on-line GC-MS the relative retention times of eluted peaks have been shown to give correct identification if used in conjunction with all detected peaks between masses 41 and 300.

Methods have been developed to interpret the spectra of incompletely resolved GC fractions (84, 85, 86) by looking at all mass spectral peaks which maximize at the same time. Where coincidence in the elution of compounds occurs, a reverse search technique must be used (87) where the library spectrum is allowed to be embedded in the unknown spectrum. Additional peaks in the unknown, therefore, do not detract from the calculated similarity index.

Read time GC-MS computer systems that identify and report the

unknown mixture components instead of mass spectral data have been developed. Meile et al (88) have described a temperature programmed GC high resolution mass spectrometer where despite large differences in chromatographic peak width and MS cycle time, a compromise of operating parameters was established and performance was minimally affected.

Multiple-ion monitoring techniques have also been used for real time identification (89). These systems are now being used for a large number of reference compounds with automatic utilization of GC retention data.

Extraction of mass spectra free of background and neighbouring component contributions from GC-MS data has been accomplished. It is an important computer-aided technique for identification. Dromeg et al (85) have produced spectra that can be corrected for interference from neighbouring eluants, peak saturation and column and septum bleed. It was shown that these computer manipulations allow the collection of representative mass spectra from GC-MS data of complex mixtures on a routine basis.

2.2.2 Chemical Reaction Techniques

The capability of GC and GC-MS can be exploited more fully if additional chemical information about a separated component is obtained. A number of functional group reactions based on chemical qualitative analysis such as hydrogenation, hydrogenolysis, ozonolysis, dehydrogenation and chemical subtraction may be used. With the small amounts of materials recoverable from single peaks on capillary columns, identification by other micro spectroscopic techniques is only sometimes possible and therefore the use of these ancillary chemical reactions is of importance for the identification of components resolved by gas chromatography.

Many variables including catalyst activation procedure, purity of reactant gas, amount and composition of samples injected, may influence

these reaction techniques. The use of controls that duplicate closely the qualitative and quantitative nature of the sample can give a greater level of confidence in results from these techniques.

Hydrogenation

By utilizing hydrogen as the carrier gas, or as a component of the carrier gas, with a nickel or palladium catalyst in an offline or on-line reactor, the existence of carbon-carbon double bonds and other structural features of unsaturated components may be determined.

Hydrogenation has been described as a pre-column technique for pure samples or simple mixtures (90) or as a post column technique in conjunction with MS for the analysis of complex mixtures (91).

The principle advantage of the technique is the availability of supplementary information for many unsaturated compounds that may contain cis or trans double bonds, branched or cyclic structures and conjugated structures that would otherwise be difficult to detect by GC retention data alone (92).

Franc and Kolovskova (93) have demonstrated the technique of pre-column hydrogenation as a method for the determination of the degree of saturation of a compound. Retention volume data were used for the characterization of each peak before and after hydrogenation. Other pre-column methods have been developed by Beroza and Sarmienta (90) who used hydrogenation as an analytical technique for a wide range of compounds such as alcohols, amides, amines, esters, and halides. They developed a device which could be used as either a post oven or injector hydrogenator to provide comparative data before and after hydrogenation.

On-line pre-column hydrogenation coupled to MS has been successfully used as a derivatization technique by Henneberg et al (42). The methodology provides additional information for compounds that can not be identified by MS alone. The identification of the different carbon

skeletons of the saturated compounds can be supported by their retention index increments.

Hydrogenation of components after GC separation but before entry into a Mass Spectrometer was first proposed by Lindeman (94) and has application in the analysis of complex mixtures. If the compound separated is passed into the mass spectrometer with or without hydrogenation, the number of double bonds can be determined from the difference in molecular weights of the parent ions. This allows hydrogenation to be used for the analysis of mixtures that may exhibit hydrogenated peaks at the same retention times as the original compound. A limiting factor is, if hydrogenation is incomplete, then interpretation of the superimposed spectra of reaction products and unchanged material creates difficulties. Another disadvantage is due to the range of reactivity of unsaturated compounds, for which a single set of conditions to fully hydrogenate a group of compounds separated by GC is impracticable.

Other applications of on-line hydrogenation have been demonstrated by Kuzmencio (95) who identified fatty acid methyl esters using capillary GC. Complicated hydrocarbon mixtures have been analysed by Reaction GC-hydrogenation (96). Mounts and Dutton (97) have reported vapour phase hydrogenation for the analysis of fatty acid esters.

Off-line hydrogenation has been proposed by Murray et al. (98) who collected separated components from the stream splitter at the GC-MS interface concurrent with the recording of their mass spectra. A similar technique has been used by Cronin et al (99) who collected compounds on palladium coated traps allowing the sample to be hydrogenation without further handling, and the reaction products to be analysed by MS.

Ozonolysis

Ozonolysis for the determination of the position of carbon-carbon double bonds has been combined with GC and GC-MS to give structural information on nanogram amounts of organic compounds. It has been found

applicable for the determination of alkylidine groups in alcohols, esters, amides, halogenated compounds, aliphatic, aromatic and heterocyclic compounds (100). The methodology uses ozonolysis of the compound, cleaving of the resulting ozonide by reduction, oxidation, or pyrolysis, and identification of the resulting products by GC or GC-MS.

Cronin and Gilbert (99) have used ozonolysis as a technique complementary to hydrogenation, characteristic colour reactions and MS, for structural identification. They have also outlined problems associated with on-line ozonolysis in a GC-MS system, and proposed an off-line system where nanogram amounts of compounds eluted as single peaks from capillary columns were trapped on columns containing an inert support. Trapped compounds were then ozonized, cleaved by heating, and the resulting carbonyl compounds then identified by GC-MS. Other invetigators in the field of natural products (101, 102) have been able to distinguish between acidic and aldehydic products resulting from ozonolysis by the addition of a short pre-column of zinc oxide for the selective removal of acids.

Carbon Skeleton Chromatography

This on line technique catalytically removes from each compound in the injected mixture all functional groups containing oxygen, nitrogen, sulfur and halogens to give the equivalent hydrocarbon skeleton and/or its next lower homologue. This basic skeleton is considered (92) to be a simple and logical starting point for the structural determination of a compound. The method has found use as an identification technique used in conjunction with GC for nanogram amounts of compounds and degradation products of naturally occurring materials (103-106).

When carbon skeleton chromatography is used in conjunction with Kovats Indices a more positive system of identification may be obtained (107). This is due to the fact that the index of the carbon

skeleton is independent of the stationary phase. With many compounds, especially terpenes, the complex mixture of reaction products may necessitate the use of MS data in addition to retention indicies so that carbon skeleton chromatography may be used to advantage.

Beroza and Inscoe have reviewed the application of carbon skeleton chromatography for the identification of trace compounds in natural products (92). They also undertook a systematic study for the structural determination of the carbon skeleton products that result from a wide range of organic compounds (105).

Carbon skeleton chromatography has also been applied extensively to the analysis of hydrocarbons. Kulemans and Vogue (108) have catalytically dehydrogenated C5-C8 hydrocarbons to yield the carbon skeleton. It was, however, found by Rowan, that with hydrocarbons the dehydrogenation process is complicated by the simultaneous production of many side products. These difficulties have been overcome by Klesment (110) who proposed using a carrier gas containing only 5 to 30% hydrogen which still enabled alkenes to be quantitatively saturated.

Subtractive Reactions

Chemical and physical subtraction techniques may be used for the removal of specific functional groups of compounds in complex mixtures. Comparison of chromatograms before and after reaction yields additional information on reacted compounds. Subtraction may be carried out by pre-injection techniques or by on-line methods.

The ideal properties of a subtraction column have been proposed by Haken et al (111) and extensive reviews of the techniques have been undertaken by Beroza and Inscoe (92), Beroza and Coad (112), Leathard and Shurlock (113) and Berezkin (114). It must, however, be emphasised that information from subtraction techniques must not be overinterpreted due to the number of variables that may influence them such as activation procedure, contaminants in carrier gas, and size and composition of

sample injected.

With on-line subtraction a reactor may be used either before or after the analytical GC column. Pre column techniques have been shown to have advantages (113) if the extracted components can be regenerated and rechromatographed enabling a comparison of the two chromatograms. Regeneration may be difficult and it is often necessary to inject two samples, i.e. with and without subtraction (115). An alternative technique when subtraction is irreversible is to use two detectors rather than two samples and use subtraction in a post column mode. This technique is considered the superior method as any irreproducibilities of injection are avoided (113).

Physical subtraction techniques for the identification of hydrocarbons using molecular sieves has been widely used and the adsorption characteristics of a wide range of compounds on these materials are known (115-120). It has only found application for the subtraction of straight chain n-alkanes and olefins from hydrocarbon mixtures. Molecular sieves do, however, remove other low molecular weight compounds and this lack of specificity limits their use.

Saturated primary and secondary alcohols may be quantitiatively subtracted using 5% boric acid coated column packing. Tertiary alcohols are not subtracted as they do not form trialkyl borates nor are alcohols subtracted that have a double bond in a position α to a secondary hydroxyl group (121-123). It was shown that primary and secondary alcohols are retained as borate esters which may eventually elute as broad peaks whilst tertiary alcohols may dehydrate to olefins. A lower concentration of boric acid (0.5%) has been used and found not to cause dehydration allowing their differentiation from other alcohols (122, 125). With the higher concentration of boric acid, some epoxides and aldehydes may also be subtracted resulting in possible difficulties in

interpretation.

Free fatty acid phase (FFAP) has been investigated for the subtraction of aldehydes (126). However, with aldehydes that are branched at the α carbon atom as much as 20% may elute unchanged (122). Some epoxides were also found to be subtracted. Withers (100) has examined its use as an aldehyde subtractor and found it unsatisfactory because of its low capacity, deterioration on keeping, and resultant large variations in retention times of non aldehydic groups.

Bierl (122) found that by the formation of a Schiff base with an amine, aldehydes were able to be subtracted from complex mixtures. The most suitable reagent tested was o-dianisidine and with the exception of cyclohexanone, ketons were not affected. A disadvantage is that with o-dianisidine, bleed was excessive over 175°C.

For the determination of epoxides Bierl (122) used a short post column reactor containing 5% phosphoric acid on chromosorb W. and found it removed up to 95% of the compound. It was concluded that subtraction by it does not necessarily mean that an epoxide is present but should be considered as a confirmatory test. Sodium metabisulphite has been found to be an acceptable subtractor for both aldehydes and ketones (111), although it does cause some retardation of compounds of boiling point in excess of 150°C due to physical adsorption effects. Most carboxylic acids may be subtracted on a pre column if 10% zinc oxide of a GC support (92). An exception was α substituted acids which were only partially subtracted. It was also demonstrated that some alcohols and phenols were removed.

On-Column Derivative Formation

Peak shifting techniques involving the formation of derivatives on a GC column, where volatile pure isolated compounds are converted to volatile derivatives have been described by Langer and Pantages (126). It may be applied to any compound that is sufficiently volatile to be

eluted from a GC column and which may react to form a volatile derivative. The retention data of the derivative increases the certainty of identification and it may be regarded as a technique analogous to dual column chromatography.

Typical applications of this methodology have been in the determination of alkaloids and steroids (127) by acylation, alcohols by conversion to acetates,(128) and on column esterification of sesquiterpenoids (129).

Vessel and Syringe Reactions

If a reaction proceeds too slowly in a GC system it may often be carried out in a reaction vessel prior to injection. The volatile reaction products may then be chromatographed by diversion of the carrier gas through the vessel.

The technique has been successfully used for the quantitative measurement of some functional group classes, including active hydrogen (130, 131) and primary amino group (132). These and other vessel reactions have been reviewed (92).

Hoff and Feit (133) have classified compounds according to reactions in a GC syringe. The vapours of the compounds were reacted to produce derivatives which were injected directly into a GC. These workers found that carbonyl compounds could be detected and ethers, olefins and aromatic hydrocarbons could be differentiated. Other applications of this technique have been reviewed (113, 132).

Pyrolysis

Pyrolytic degradation techniques coupled with GC have been used extensively for the examination of sub-microgram quantities of materials of relatively low volatility that do not chromatograph easily. An advantage of this method is that it may be used to produce a fingerprint chromatogram suitable for characterization. The reliability of the

identification may be greatly improved if mass spectrometry is used in conjunction with the pyrolysis-GC system (72).

Several techniques for pyrolytic degradation have been developed, including filament, boat, quartz tube, Curie point, photochemical and laser techniques, and the methodology and applications have been reviewed biennially (20, 134-136).

Spot Tests

Conventional methods of qualitative analysis relying on specific functional groups to form colour reactions, may be adapted for use with GC enabling peaks to be characterized into particular classes of compounds (137-140).

Walsh and Merritt (137) used a stream splitter to facilitate the classification of peaks containing any one of eleven functional groups. The simplicity of the technique enables it to be used as a method for peak identification, and the use of recently introduced trapping procedures (141) may permit its extension to small bore glass capillary eluents.

Casu and Cavollotti (138) have developed a method where a thin layer strip is impregnated with reagent, then passed over the column effluent at the same rate as the detector recorder. It was found that some peaks could be attributed to overlap of a minor component containing the functional group of a major component. Except for GC-MS-computer methods, there are few other techniques that may be used to differentiate overlapped peaks so readily.

Column Chromatography, HPLC, and TLC in Combination with GC

Simple pre-fractionation techniques have been used for the separation of complex mixtures before Gas Chromatography. The fractionation of complex mixtures such as leaf oils(142) has been undertaken using column chromatography and was shown to be better than preparative GC.

Unfortunately acid catalysed isomerization of terpenes on some chromatographic adsorbents occurs and most workers have been deterrred by this procedure.

Von Rudloff (142) has demonstrated the applicability of column chromatographic pre-fractionation to terpene analysis and has concluded, after studies with several oils, that isomerization may be largely prevented by the use of deactivated silicic acid.

Alumina adsorbent has similarly been shown to catalyse terpenoid isomerization (143) and isomerization of epoxides to alcohols together with some side reactions.

The combination of TLC and GC may be utilized to produce a more complete separation of all components with a particular functional group within a complex mixture. By the use of silver nitrate/silica gel TLC, cis- and trans- unsaturated isomers may be differentiated before GC separation.

Coupled GC/TLC has been used by Nigam et al (145) for the simultaneous determination of terpenes in mint oil. Combined GC/TLC has also been described by Kaiser (146) and Janak (147), however several of the separations obtained by this technique can also be achieved by GC using the appropriate liquid phase.

Whilst HPLC is usually the method of choice for the separation of non volatile compounds, it may also be used to advantage as a pre-fractionation technique. Fractions of interest must be eluted from the column and extracted with a solvent compatible with the gas chromatographic separation conditions. This technique is beginning to have application in studies of flavour components and commerical units coupling HPLC and GC have been described (144).

2.2.3 Gas Chromatography - Infrared Spectroscopy

Infrared spectroscopic techniques (IR) may provide further information

for structural assignment of compounds eluted from a GC. Even though IR is widely applicable to most compounds, it does not record the full vibrational contributions from bonds such as C-C, C=C, C≡C and C-H. To adequately measure vibrational contributions from these bonds, the Raman scattering spectrum must also be recorded over the same energy range.

The IR spectrum may be used as a fingerprint method of identification, based upon a file of reference spectra, and also as a means of functional group determination. Even though group frequency data interpretation may lead to partial structural characterization, the data may confirm or be supplementary to that obtained from MS and other spectroscopic studies. In this respect IR can give structural information that may not be obtained by other techniques, such as the specific environment of the carbonyl group, the stereochemistry of the hydroxyl moiety, the presence of a gem-dimethyl system, or the type of aromatic substitution.

Recent discussion on structural identification by IR spectroscopy have been included in recent texts (148) and comprehensive reviews of on line GC-FTIR (149) and non dispersive IR sampling techniques have been published (150).

Off-line Collection of GC Fractions for IR Identification

The collection of fractions for IR analysis requires efficient sample manipulation techniques in order to provide microgram amounts of pure fractions. Many different collecting devices have been described, however few are effective for compounds of differing volatilities and for collection in the sub-microgram range. These inherent problems of isolation and collection have been well reviewed (142, 151, 152).

Although these fraction collection techniques are laborious and limited to the use of low resolution columns, it is the only method available for the collection of GC effluents present in sufficient

quantities to record an IR spectrum when sophisticated on-line GC-IR and GC-FTIR instruments are not available.

The collection of fractions for the measurement of solution spectra is preferred as the problems associated with sample transfer are reduced (153, 154). Simple condensation traps have been developed (155) which have demonstrated the efficiency of thin walled glass capillaries for collection of microgram amounts of eluents. In order to reduce fogging of the sample to a minimum, Schlenk and Sand (156) heated a section of the capillary.

Techniques have been reported for trapping fractions on potassium bromide which is then pressed into a pellet (157). It was shown that spectra could be routinely obtained on 20 μg of sample (154). If the sample was first isolated in a glass capillary and solvent washed onto a potassium bromide disc, a sensitivity increase of an order of magnitude was obtained (158).

Collection of high boiling components directly onto a membrane filter has been investigated. An advantage of this method is that it requires no sample transfer step as the filter may be placed directly in the beam of the spectrophotometer. This technique has, however, been found to have low trapping efficiency (150).

Bierl and Beroza (151) have developed a sampling device which is effective for components in the low nanogram range that also provides for the efficient transfer of trapped compounds without a solvent. Fractions are collected on a short column of gas chromatographic packing and transferred to an IR cell. Good recoveries are obtained at the 10 ng level.

Although it is possible to collect low boiling liquids by cold trap techniques, μℓ fractions are difficult to retain during IR measurement. Galaway et al. (159, 160) have routinely collected and identified 10 μℓ

of gaseous components in a heated microcell with a 1mm path length, while good spectra have been recorded from microgram quantities in gas cell traps of volumes down to 45 μℓ (161).

As these sampling techniques only allow for the collection of microgram amounts, off-line IR identification of GC fractions has limited applicability when compared with on-line GC-FTIR and GC-MS techniques.

On-line GC-IR Techniques

The combination of GC with dispersive IR instruments has been used for many years for the direct recording of spectra. Various systems have been developed (162-164), however, they suffer from loss in quality of the spectra due to slow scan speeds of approximately 30 seconds. This combination can only be considered adequate where only a few chromatographic peaks are of interest.

These instrumentation problems have been overcome by the introduction of Fourier-transform spectrometers giving a high throughput and allowing a spectrum to be recorded in 0.25 seconds. This advantage allows a chromatographic peak to be scanned several times and multicomponent peaks to be deconvoluted.

With this technique the GC is interfaced by a heated light path optimized to the anticipated average GC peak volume (149) resulting in the recording of a vapour phase spectrum (165). While relationships between structure and IR absorption wavelengths are well documented for solution spectra, significant differences may exist in the vapour phase (163). Although spectral shifts for some bands of up to 20 cm^{-1} (164) have been noted, Welti (163) has demonstrated the advantages of direct collection and examination in the vapour phase.

At present the sensitivity of GC-FTIR is about 10 ng per component, which is less than most GC detector systems. It has been predicted (162)

that an ultimate limit of 1 ng may be attained, thus sensitivity may remain a disadvantage in comparison to GC-MS.

2.2.4 Raman Spectroscopy

Raman spectroscopy complements and extends structural information available from IR spectroscopy, resulting in a more complete view of the vibrational behaviour of the molecule (166). The technique is of particular value where the symmetry of the dipole moment restrictions of the molecule do not permit the infra-red spectrum to represent the full vibrational spectrum.

Raman spectroscopy has several advantages over IR (154) in that simpler instrumentation allows continuous measurement from 0 - 4000 cm^{-1} enabling location of torsional oscillations around 50 cm^{-1} which distinguish conformers. In addition the Raman spectrum of water does not interfere with most measurements, whilst the intensity of the Raman bands varies linearly with concentration. As a result more distinctive fingerprint spectra are often obtained (166).

The introduction of laser excitation has enabled many fluorescent problems to be overcome, and also allows multiple passing techniques permitting an increase in the signal to noise ratio for liquids of weak scattering power (154). The collimated nature of laser energy allows focussing for excitation on small sample volumes enabling simpler handling techniques and often smaller sample size requirements than with IR analysis.

For off-line GC-Raman spectroscopic techniques, the co-axial viewing method enables radiation to be more efficiently collected from small bore capillary tubes using the principle of total internal reflection. As the same glass capillary tubes may also be used to collect GC fractions (167), the need for sample transfer with associated losses is overcome. The greater efficiency of this technique allows spectra to be obtained from sub microlitre amounts.

Other micro sampling and Raman measurement systems have been developed that give spectra with similar detection limits to that of IR. A disposable glass capillary cell (168) made by blowing a sphere on a capillary tube and silvering one side gave good spectra on a 0.04 μℓ sample. Microsampling systems (154) using 0.5 mm ID capillaries for fraction collection, which were then sealed and centrifuged, gave interpretable spectra on 0.1 μℓ of sample.

On-line identification of GC fractions has been described using a Raman multichannel spectrometer (169) focussed on the exit slit of a capillary column. Detection limits similar to FTIR have been accomplished, however little interest has been shown in the development of this complementary on-line GC technique.

2.2.5 Nuclear Magnetic Resonance Spectroscopy (NMR)

NMR may be used to give structural information regarding the presence of particular atomic groupings, relative numbers of NMR-active nuclei in different environments, the structural relationship of the different atomic groupings and the stereochemistry and conformation of the molecule. In comparison to the previously mentioned spectroscopic techniques, the principle use for NMR is in structural elucidation of new compounds or for identification where no recorded spectra are available.

The combination of recent computer techniques with high magnetic field and pulsed Fourier transform instrumentation has enabled NMR to have the same capability with microquantities as IR spectroscopy.

Off-line collection of GC fractions for NMR analysis may be accomplished using similar collection techniques as previously reviewed for IR sampling. Hall (170) has found the most applicable to be sample condensation directly into a glass microcell, directing the effluent through cooled carbon tetrachloride and trapping on a tube filled with column packing. Quantitative recoveries were also obtained from a

technique which trapped fractions in a packed tube followed by vacuum-line transfer directly into a spherical microcell.

The minimum amount of sample required to produce an NMR spectrum depends largely on the magnetic properties of the element of interest, the limitations of the instrument and the sensitivity enhancement technique used for investigation of microquantities.

Pulse methods allow a large decrease in experimental time and a reduction in minimum sample size requirement. Fourier-transform techniques, especially when used with multiphase instrumentation, provide a sensitivity factor increase of one hundred or more and a considerable time saving. This combination of advantages has allowed coupling of GC directly to an NMR spectromer (171) where spectra were obtained on fractions flowing through a magnetic field; the beginning and termination of the pulse sequence being determined by the thermal conductivity detector.

CHAPTER 3

3. Experimental

3.1 Isolation of Essential Oils from Atherosperma moschatum and Leptospermum lanigerum

Fresh foliage was collected from the Tasmanian Botanical Gardens during January 1980 and steam distilled to yield the essential oil components of both oils. The distillate was extracted with ether and the extracts were evaporated under a stream of nitrogen to give a yield of 3.4% for A. moschatum and 4.2% for L. lanigerum.

This extraction procedure is in contrast to that used by some workers (172, 173) in which the water soluble extracts were not extracted, therefore not providing a true composition of the oil.

3.2 Dual Packed Column Identification of Components in Oil of A. moschatum by Relative Retention Data

3.2.1 Introduction

Relative retention data have often been investigated for the identification of components in essential oils (142). The possibility of several components exhibiting the same retention on a given column is very real. Increased certainty of identification can be given to identifications assigned on the basis of relative retentions using two dimensional chromatography (174), i.e. separation on one column followed by a second separation on a dissimilar liquid phase. In many analytical situations, dual column retention data have been adequate for the identification of many compounds present in essential oils.

In the choice of liquid phases, carbowax 20M has been widely used in both analytical and preparative applications due to its polar nature. Although it is less polar than PEG polymers of lower molecular weight, it exhibits better temperature stability and may be used to 220°C without excessive bleeding; OV-17 phase was selected from the literature as a non-polar phase for the separation of terpenes according to their boiling points.

A dual-packed column low resolution GC system CW-20M, OV-17 was established in order to undertake preliminary investigations of the oil of A. moschatum.

3.2.2 Experimental

(i) Preparation of columns

From the literature it was determined that the most common loadings used were 3 to 5% of liquid phase on Gas Chrom Q support 100-120 mesh.

Empty 2m x 4mm ID glass columns were washed with chromic acid, distilled water, ethanol and ether then dried under a stream of nitrogen. The columns were then silanized to block active sites by rinsing with a 5% solution of dimethyl dichlorosilane (DMCS) in toluene, rinsing with toluene and ethanol then drying under a stream of nitrogen.

Coated solid supports were prepared by mixing a slurry of the support with the appropriate solvent containing the liquid phase. The solvent was then evaporated off in a stream of nitrogen while the flask was rotated over a warm water bath.

Columns were packed with the aid of vacuum (approximately 250 Torr mercury) and conditioned in the GC oven by temperature programming at 0.5°C per minute over the full operating range of the particular phase. During conditioning,columns were tested for reproducibility by injection of various terpene standards until a reproducible chromatogram was obtained.

(ii) Preparation of terpene standards

Pure terpenoids were isolated by preparative GC and their structures verified by micro IR spectrometry. These compounds were then co-injected with reference standards α-pinene at 80°C and camphene at 130°C onto both analytical columns. The relative retention times (RRT) of these terpenoids are shown in Tables 1 and 2.

(iii) Analytical GC operation

Chromatograms for calculation of RRT data were obtained using a Varian 3700 GC with dual flame ionization detectors. The following conditions were used:

Column temperature :	80°C and 130°C isothermal
Carrier gas :	high purity nitrogen 30 ml/min.
FID gas :	hydrogen 15 psi 40 ml/min.
	air 50 psi 30 ml/min.
Inlet temperature :	205°C
Detector temperature :	220°C
Attenuation :	32×10^{-8}
Chart speed :	1 cm/min.
Injection :	0.2 µℓ

Retention times and areas were measured with a Shimadzu Chromatopac E1A plotter integrator.

(iv) Determination of RRT data for Atherosperma moschatum

Relative retention data were calculated for each component of the oil using the same conditions and techniques as previously described. Isothermal chromatograms were run on both phases at both temperatures by co-injecting the RRT standard (α-pinene or camphene) with 0.2 μℓ of the oil.

A temperature programmed run of the whole oil was then undertaken on each phase, Figure 1 and Figure 2, and the tentative identification corresponding to each chromatogram is listed in Tables 3 and 4.

The possible identification of components present was calculated from Tables 3 and 4 and is listed in Table 5 together with percentage composition.

Table 1

Relative retention times (mins) of terpenes injected as liquids on two dissimilar columns, reference α-pinene, 80°C

	5% Carbowax 20 M	5% OV-17
Terpenoid	RRT	RRT
camphene	1.17	1.17
β-pinene	1.37	1.48
Δ-3-carene	1.65	1.78
α-phellandrene	1.67	1.75
β-myrcene	1.71	1.56
(-)-limonene	2.05	2.10
(+)-limonene	2.10	2.10
cineole	2.36	2.41
β-phellandrene	2.68	2.34
ρ-cymene	2.81	2.39
terpinolene	3.07	3.45

Table 2

Relative retention times of terpenoids injected as liquids on two dissimilar columns, reference camphor, 130°C

	5% Carbowax 20 M	5% OV-17
Terpenoid	RRT	RRT
(+)-linalool	0.92	0.60
caryophyllene	1.14	2.73
bornyl acetate	1.16	1.65
terpinen-4-ol	1.21	1.01
pulegone	1.53	1.62
borneol	1.71	0.96
α-terpineol	1.74	1.08
bornyl acetate	1.90	2.30
nerol	2.64	1.28
anethole	2.75	2.13
geraniol	3.22	1.47
safrole	3.31	2.27
ethyl methyl ether	5.35	7.80

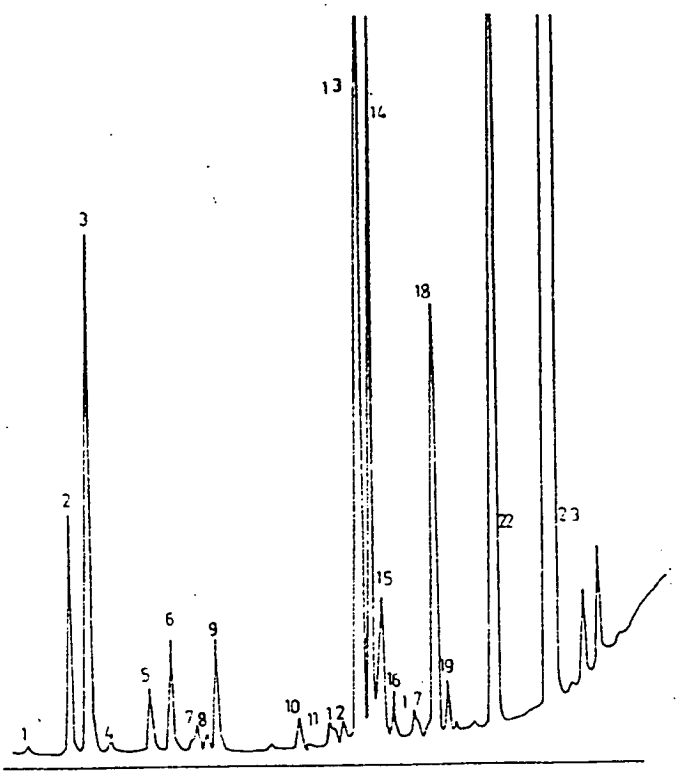

Figure 1. Temperature programmed chromatogram of whole oil of Atherosperma moschatum on a CW-20m packed column, temperature programmed 50°-200°C at 5°C per minute.

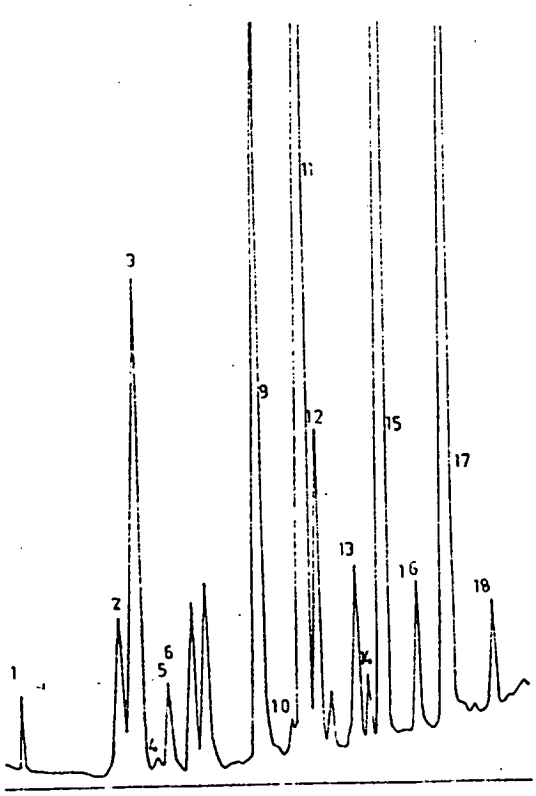

Figure 2. Temperature programmed chromatogram of whole oil of Atherosperma moschatum on a OV-17 packed column, temperature programmed 50°-200°C at 5°C per minute.

Table 3

Tentative identification of components in whole oil of *A. moschatum* CW-20m, from Figure 1.

Peak No.	Tentative Identification	% composition
1	unknown	tr
2	α-pinene	0.9
3	camphene	2.9
4	β-pinene	0.1
5	Δ3-carene or α-phellandrene	0.4
6	β-myrcene	0.7
7	limonene	0.1
8	1,8-cineole	0.1
9	α-phellandrene	0.6
10	unknown	tr
11	unknown	tr
12	unknown	tr
13	linalool)	14
14	camphor)	
15	caryophyllene ? bornylacetate ?	0.6
16	caryophyllene, bornyl acetate terpinen-4-ol	0.4
17	pulegone terpinen-4-ol	0.2
18	borneol	1.9
19	unknown	0.4
20	unknown	tr
21	unknown	tr
22	safrole+geraniol	11.5
23	eugenol methyl ether	63

Table 4

Tentative identification of components in whole oil of *A. moschatum*, OV-17 phase, from Figure 2.

Peak No.	Tentative Identification	% composition
1	unknown	tr
2	α-pinene	1.0
3	camphene	3.1
4	β-pinene	0.1
5	β-myrcene	0.4
6	Δ-3-carene, α-phellandrene	0.7
7	limonene	1.1
8	cineole ? ρ-cymene ? β-phellandrene ?	0.1
9	linalool	4
10	? unknown	
11	camphor ? borneol ?	12.6
12	nerol ? borneol ?	
13	pulegone	0.8
14	unknown	
15	safrole	11.5
16	bornyl acetate	0.5
17	eugenol methyl ether	51

Table 5

Identification of components in whole oil of A. moschatum by RRT on two dissimilar columns.

Compound	% composition CW-20m	OV-17
unknown	tr	tr
α-pinene	0.9	1.0
camphene	2.9	3.1
β-pinene	0.1	0.1
β-myrcene	0.7	0.4
Δ3-carene or α-phellandrene	0.4	0.7
limonene	0.1	1.1
1,8-cineole or cymene or β-phellandrene	tr	0.1
β-phellandrene	0.6	
unknown	tr	tr
unknown	tr	tr
unknown	tr	tr
linalool	14	4
camphor		12.6
borneol		
pulegone		0.8
safrole	11.8	11.5
bornyl acetate	0.6	0.5
eugenol methyl ether	53	51

3.2.3 Results and Discussion

From Tables 3 and 4 a tentative identification of fifteen compounds present was made (Table 5). Some components were found to be in agreement with earlier work of Scott (175) who investigated this oil and found pinene (15.2%), camphor (15-20%), and eugenol methyl ether (50-60%). Ayling reported in later work (176) finding α-pinene (1.3%), camphene (5%), γ-terpinene (<0.1%), ρ-cymene (0.4%), linalool + camphor (18.2%), α-terpineol (3.3%), safrole (13.8%), eugenol methyl ether (49.5%) and tentatively identifying β-pinene, myrcene, α+β-phellandrene, 1,8-cineole, terpinolene, terpinen-4-ol and bornyl acetate.

Differences in concentration may be due either to the error factor introduced by using peak height as a criterion for concentration or due to component concentration changes within the species (177, 178, 179). The differences found in minor component composition can not be commented on as these were only tentatively identified.

Ambiguities in the identification of compounds listed in Table 5 are: limonene, 1,8 cineole, ρ-cymene, β-phellandrene and pulegone. All or only some of these compounds may be present due to the fact that low resolution columns were used resulting in compounds co-eluting on one phase and separating on a phase of different polarity.

Other liquid phases could have been tried in order to obtain a more complete separation, however, because of the great diversity of terpenes and the fact that most oils contain monoterpenoid constituents as alcohols, esters, ethers, eugenols and many other steam volatiles, each group would have its own resolution problems.

Several aromatic ethers such as safrole and methyl eugenol (180) have also been shown to make resolution and separation of other compounds difficult.

Since complete resolution on packed columns is difficult to achieve and since terpenes of different structure may have identical retention times on a given phase, co-injection of standards did not overcome these problems. For comparative studies on mixtures that have already been identified this comparison of retention and co-injection may be a valid means of characterization, for example, in chemosystematic studies (181).

The use of high resolution SCOT or WCOT columns would help in overcoming these resolution problems.

Unknown components found could possibly be identified if a greater number of standards was available for comparison. Reliable literature data for RRT data comparison of unknowns are limited. This is mainly due to difficulties in inter-laboratory reproducibility of gas flow rates, batch differences of phases, reactivities of solid supports and the limited control of such parameters as temperature and injection technique. Even if literature values were available, they should only be used as a tentative guide (182).

3.2.4 Conclusions

Relative retention data on dual columns did not provide a convenient and reliable means of identification of all the individual components in a complex essential oil. Data was only reproducible on the same columns and instrument variations may occur (183). It can only be considered as a preliminary investigatory technique.

The methodology did however demonstrate the simplicity of operation and calculation which may enable a rapid tentative identification of the major components in an essential oil.

It must be concluded that for a more positive identification, more complete resolution of components is required, i.e. the use of open bore columns, together with a more universal retention system such as Kovats Indic es in order to overcome the problem of a lack

of suitable standards.

3.3 Isothermal Kovats Indices

3.3.1 Introduction

Isothermal Kovats Indices (I) have been proposed as a means of identification of components of essential oils and applications have been regularly reviewed (184, 185). The identification system records the retention index I utilizing the relationship existing between elution time of a compound and a known homologous series of standards. The method should show greater precision when compared with relative retention time identifications as standards may be chosen in such a way that their retentions are similar to the compounds to be eluted.

The lack of reliability of previously investigated retention data due to the limited separation efficiency and other column effects has resulted in some investigators (9) using high resolution WCOT and SCOT columns for the investigation of essential oils.

In order to overcome the inadequacies associated with the packed column relative retention system, dual column high resolution isothermal Kovats Indices were investigated as an identification technique for the components in essential oils.

3.3.2 Experimental

Dual SCOT columns of OV-101 and Carbowax 20M stationary phases were used for recording Kovats Indices. This combination was selected as recent literature suggests the use of these stationary phases for capillary separation of essential oils (184).

Each column was installed in a Varian 3700 GC equipped with dual FID's and SGE injector systems. A splitter injector system was chosen because on any column there is a limit to the sample size it may handle without becoming overloaded. This becomes important

especially when analysing concentrated mixtures such as essential oils.

The splitter vapourizer tube was packed with silanized glass beads in order to assist with the even vaporization and effective mixing of the sample before entering the column.

Hydrogen carrier gas was used because higher velocities in capillary columns do not result in a large loss in separation efficiency as occurs with nitrogen.

Capillary columns were conditioned by temperature programming from 20°C to 220°C at 1°C per minute. The column pressure was optimized at 4 to 5 psig, and the linear gas velocity calculated by successive injections of methane.

Test chromatograms were run (in order to optimize the performance of the system) by the injection of an activity mixutre containing 0.5% in chloroform of naphthalene, 2-octanone, 1-octanol, 2,4-dimethyl-aniline and 2,1-dimethylphenol.

By adjusting carrier gas flow rate and splitting ratio, the optimum number of effective plates was obtained, calculated from the formula :

$$N_{eff} = 5.54 \left(\frac{tr - ta}{w_{\frac{1}{2}}}\right)2$$

where

$w_{\frac{1}{2}}$ = peak width at half height

tr = retention time of peak

ta = dead volume

This measurement of column efficiency is dependent on the capacity ratio of the column, K, which is the ratio of hold up time of the measured peak at a measured temperature to the dead volume of the solvent. As this value should ideally be 4 to 5, the N_{eff} was therefore calculated for a compound eluting at this specified

K value.

To ensure consistency, operating conditions were checked regularly for both OV-101 and CW-20M columns using the activity mix. These parameters were as follows:

Column	CW-20M	OV-101
N_{eff}	48,700	42,700
Length	50m	55m
Type	SCOT	SCOT
ID	0.5 mm	0.5 mm
T. injector	200°C	200°C
T. detector	220°C	220°C
T. column	70°C and 150°C	70°C and 150°C
ΔP	4 psig	4.2 psig
Carrier gas	H_2	H_2
Detector	FID	FID
Gas velocity	22 cm/sec	18 cm/sec

Gas hold up times were calculated at both 70°C and 150°C by the method of Gold (23) using the linear relationship between the logarithm of the corrected retention time and the number of carbon atoms in the eluting compound. A mixture of three homologous n-alkanes were injected and the hold up time calculated by solving a series of simultaneous equations using the method of successive approximations.

Where it was feasible, a direct injection of methane was used to establish dead volume as it is suggested (184) that the use of this technique is simpler and probably as accurate. Good agreement was found for either method.

Kovats Indices standards were prepared by diluting AR grade C7-C20 alkanes (purity checked by GC) to approximately 0.4 g per

100 mls in AR chloroform.

A series of trial injections of standards was conducted to show the validity of calculating Indices from standards injected immediately before and after the sample in question, or by co-injection. As there was no difference between these two techniques, where difficulties arose from complex mixtures masking the n-alkane standards, indices were calculated from standards that were not co-injected. For all other determinations standards were co-injected.

All retention times were measured with a shimadzu Chromatopac E1A integrating plotter and for calculation of approximate solute concentration, the peak area was considered to be adequate.

Isothermal Kovats Indices (2) were calculated from gas chromatograms obtained by injecting 0.2 µℓ of sample and standard, using the following relationship:

$$I = \frac{\log T_{n(s)} - \log T_{n(z)}}{\log T_{h(z+1)} - \log T_{n(z)}} + 100z$$

where $\log T_{n(z)}$ = adjusted retention time of n-alkane with z carbon atoms

$\log T_{n(z+1)}$ = adjusted retention time of n-alkane with z+1 carbon atoms

$\log T_{n(s)}$ = adjusted retention time of eluted compound.

Indices were obtained for components of the whole oil of Atherosperma moschatum, chromatographed isothermally at 70°C (Figure 3) and 150°C (Figure 4). Literature values, compared with possible dual column RRT data identification are listed in Tables 6 and 7.

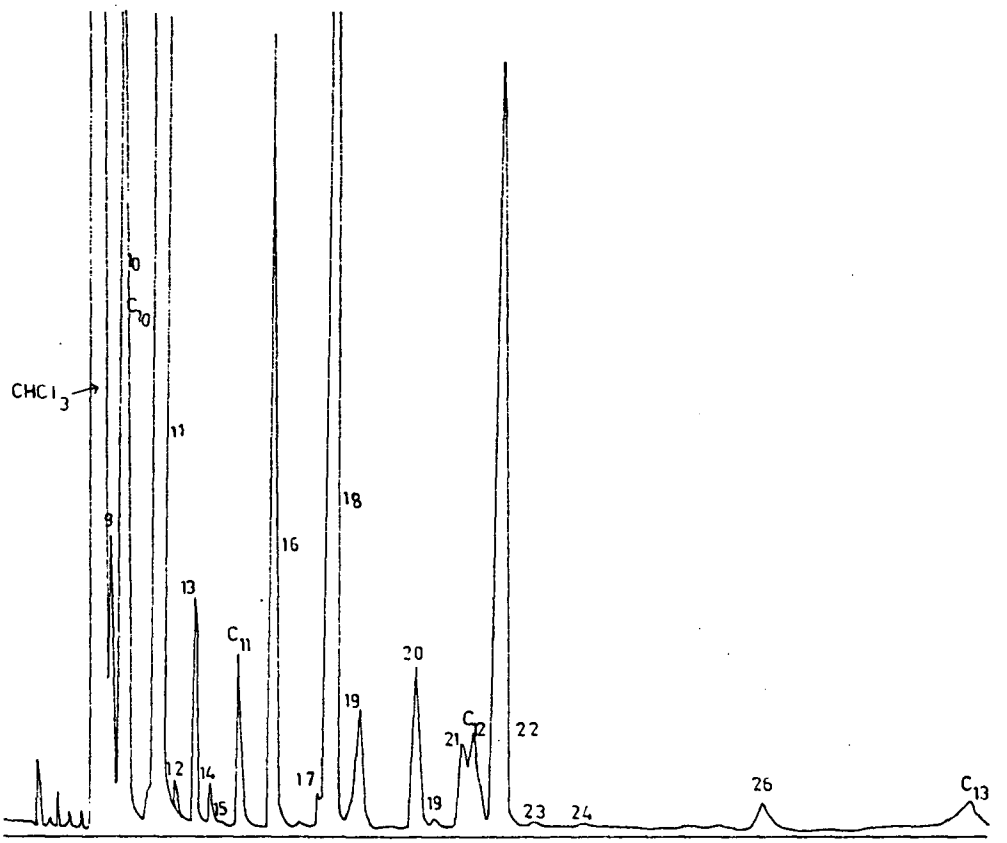

Figure 3. Isothermal chromatogram of whole oil of Atherosperma moschatum CW-20m SCOT column, 70°C, co-injected with nC_{10}-C_{14} alkanes.

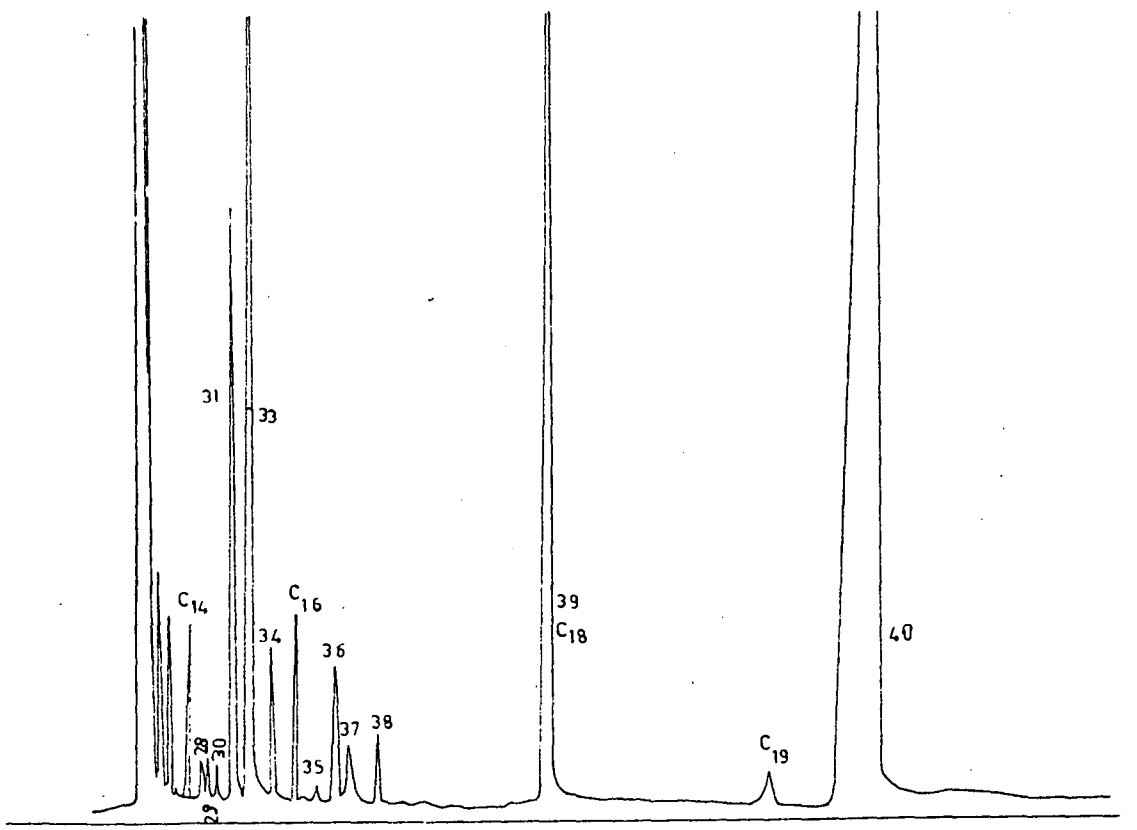

Figure 4. Isothermal chromatogram of whole oil of Atherosperma moschatum CW-20m SCOT column 150°C co-injected with nC_{13}-C_{20} alkanes.

Table 6 Isothermal Kovats Indices (I) of components of whole oil of *A. moschatum* calculated from Figure 3, gas hold up time = 4.64 min.

Peak No.	RT-GHT	I	Possible Identification by Kovats Indices (2)	Dual Col. RRT Identification
C9	1.40			
6	2.23	959	?	
9	2.62	979	?	
10	2.98	995	?	α-pinene
C10	3.10			-
11	4.14	1038	α-pinene (1038)	camphene
12	4.18	1042	?	?
13	5.17	1068	?	β-pinene
14	5.67	1080	? camphene (1066)	?
C11	6.62			
16	7.26	1120	β-pinene (1120)	
17	7.98	1133	sabinene (1130)	
18	9.05	1149	myrcene (1168)	myrcene
19	9.83	1161	myrcene (1168)	
20	11.51	1181	α-phellandrene (1170)	α-phellandrene or Δ3-carene
21	12.88	1197	?	
C12	13.21			
22	14.05	1209	limonene (1210)	limonene
23	14.96	1217	β-phellandrene (1280)	β-phellandrene
24	16.33	1228	1,8-cineole (1223)	1,8-cineole
25				
26	21.80	1266	p-cymene (1275)	
C13	27.90			

Table 7 Isothermal Kovats Indices of components of whole oil of *Atherosperma moschatum* from Figure 4, gas hold up time = 3.40 mins.

Peak No.	RT-GHT	I	Possible Identification by Kovats Indices (2)	Dual Column Identification
C13	2.19			
27	2.29	1316	?	
C14	2.91			
28	3.04	1412	fenchone (1410)	?
29	3.19	1426	?	
30	3.45	1448	?	
31	3.95	1487	linalool (1506)	linalool
32	4.19	1504	linalool (1506)	?
33	4.48	1523	camphor (1518)	camphor
34	5.19	1565	bornyl acetate (1584)	caryophyllene/ bornyl acetate
C16	5.87		-	
35	6.44	1623	terpinen-4-ol (1628) terpinen-4-ol (1628)	terpinen-4-ol
36	6.99	1644	citronellyl acetate (1645) α-terpineol (1661)	terpinen-4-ol
37	7.38	1657	pulegone (1662)	pulegone
38	8.19	1683	borneol (1698)	borneol
C18	13.08		-	
39	13.29	1804	safrole (1876)	safrole
C19	20.08			
40	22.99	1934	- (2044)	methyl eugenol
C20	29.83			

3.3.3 Results and Discussion

Capillary GC on CW-20m phase separated 40 different peaks from oil of A. moschatum in comparison to the twenty two peaks resolved by low resolution packed columns. This has had the advantage of separating co-eluting peaks that may have had an influence on relative retention time calculations.

From the data obtained (Tables 10 and 11), literature values (184) were found for eighteen peaks eluting within several index units. However, discrepancies were found in the compounds identified by Kovats Indices and the compounds previously identified by dual column relative retention time, and these are listed as follows:

Peak No.	Identified by RRT	Deviation in Kovats Indices
10	α-pinene	44
11	camphene	45
13	β-pinene	38
31	linalool	19
38	borneol	15
39	safrole	72
40	methyl-eugenol	110

The elution characteristic of these compounds were rechecked several times with respect to n-alkane standards and the data was found to be reproducible within ±2 units.

From the chromatograms, Figures 3 and 4, it was found that the greater deviation occurred at either end of the Kovats Index range, i.e. between 700 and 1200 and between 1700 and 2000 units.

Inter-laboratory discrepancies of data have been well reviewed (134, 135, 136), however anomolous behaviour of this nature has not been reported. It may be assumed that these anomolies result from a combination of physical effects such as sample injection and

splitting, the wide range of solute concentration and the chemical changes that may occur in a particular phase.

Several compounds identified by Kovats Indices were found to be in agreement with RRT identifications, i.e. pulegone, camphor, terpinen-4-ol, 1,8-cineole, α-phellandrene, and myrcene. With compounds that were tentatively identified by relative retention times, isothermal Kovats Indices could not be used as a confirmatory technique as little regard could be placed on the anomolous values found in the ranges previously stated.

3.3.4 Conclusions

Due to the discrepancies found between isothermal Kovats Indices and relative retention identification, and the anomalous behaviour of CW-20M phase, it was decided not to investigate this technique further. As the mixture investigated was extremely complex, temperature programmed dual column calculation of indices used in conjunction with spectroscopic identification would possibly be a better identification technique.

3.4 Identification by Temperature Programmed Kovats Indices on Dual Columns using Polygonal and Spline Function Approximations

3.4.1 Introduction

Due to the wide range of boiling points and polarities of compounds present in complex mixtures, many workers have suggested the use of temperature programmed Kovats Indices as a method of identification.

Theoretically, if a number of assumptions is applied, the retention times of the higher members of a homologous series are known to correlate almost linearly with the number of carbon atoms. With lower members of a series it has been shown experimentally that departures from linearity are real and reproducible (186).

Van den Dool et al. (18) have shown this non-linear relationship may be minimized if the temperature programmed Kovats Indices may be approximated to isothermal data by a polygonal relationship. Kugler et al (21) found that the use of a cubic spline function approximation produces a smooth curve through the data points resulting in a more realistic fit of data.

Jennings et al (184) have suggested that if temperature programmed data are only approximate it is still of great value and may be sufficient to confirm identification in conjunction with spectrometric data.

A dual column temperature programmed CW-20M, OV-101 SCOT capillary system was investigated for the possible use of this data in conjunction with spectrometric methods for the identification of components present in essential oils.

3.4.2 Experimental

The same analytical GC conditions were used as for the isothermal calculations except that the temperature was programmed from 50°C to 220°C at 5°C per minute. A series of n-alkane standards was co-injected with samples of the essential oils of Leptospermum lanigerum and Atherosperma moschatum in the same concentrations as previously used for isothermal calculations of Indices. Indices (I) were calculated from non-adjusted retention times using the approximation of Van den Dool and Kratz (18) where it is assumed that the points on a nearly linear curve may be approximated to a straight line by the polygonal relationship:

$$I = 100\frac{Tn\ (a) - Tn(z)}{Tn(z+1) - Tn(z)} + 100z$$

where Tn(z) = retention time of n-alkane with z carbon atoms

Tn(z+1) = retention time of n-alkane standard with z+1 carbon atoms

Tn(s) = retention time of eluted compound.

The calculation of retention indices by spline functions was undertaken by the method of Kugler et al (21) using the same retention data as for calculation by polygonal approximation. By this method calculation of data was able to be compared in order to determine if any significant differences resulted from either method.

The method of calculation of interpolating natural cubic splines for a set of data points and the construction of an algorithm for the calculation of retention indices is listed in Appendix I.

Temperature programmed chromatograms are given in Figures 5, 6, 7 and 8 for the whole oils of L. lanigerum and A. moschatum on both CW-20m and OV-101 phases. Calculated Indices by both spline functions and polygonal approximation are listed in Tables 8, 9, 11 and 12 together with possible identifications. Correlations with dual column relative retention identification are also listed for oil of A. moschatum.

3.4.3 Results and Discussion

3.4.3.1 Temperature programmed Kovats Indices identification of components present in Atherosperma moschatum.

From a comparison of Table 8 and Tables 6 and 7, little difference was found between indices calculated by temperature programming and those calculated isothermally on carbowax 20M. Temperature programming did not alter Index results by more than several units. Successive GC determinations were undertaken and Indices were found to be reproducible on this column to within 2 units.

Indices were calculated for components eluting on OV-101 phase (Table 9) and possible identification of components according to

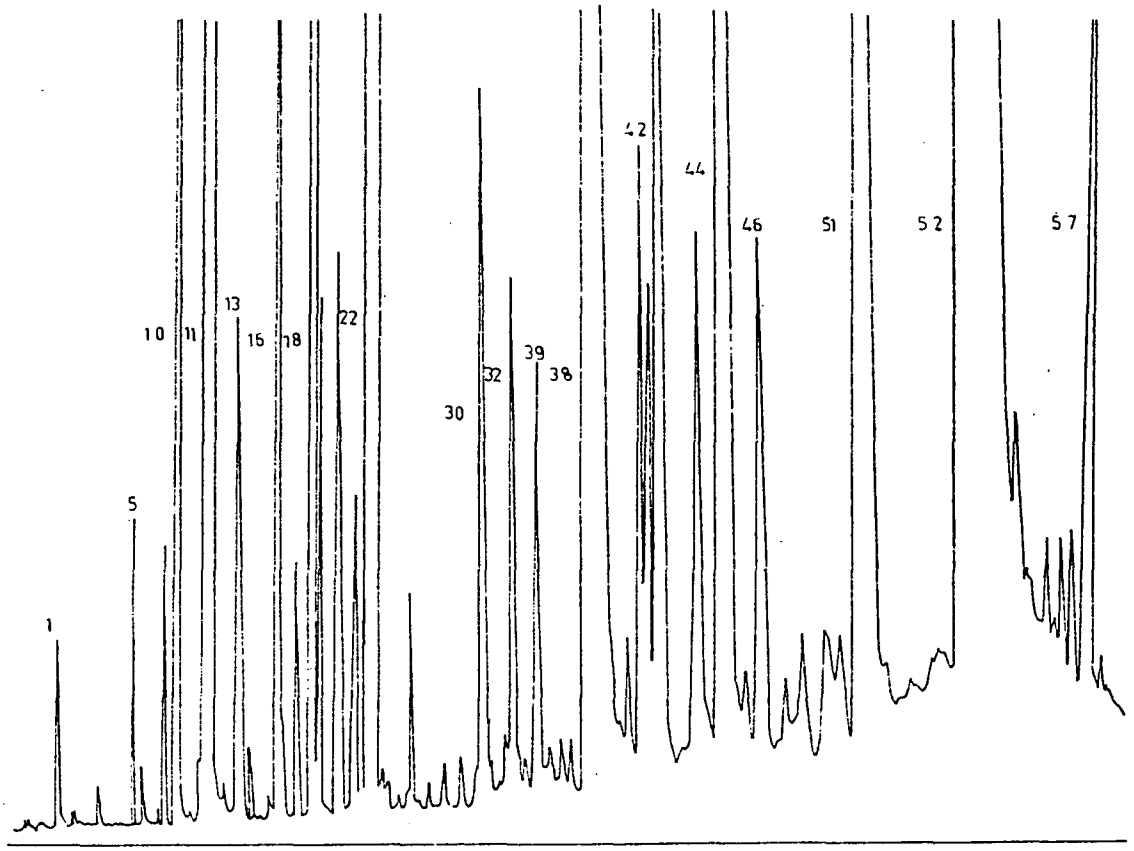

Figure 5. Chromatogram of whole oil of Atherosperma moschatum, CW-20m SCOT column temperature programmed 50°-200°C at 5°C per minute.

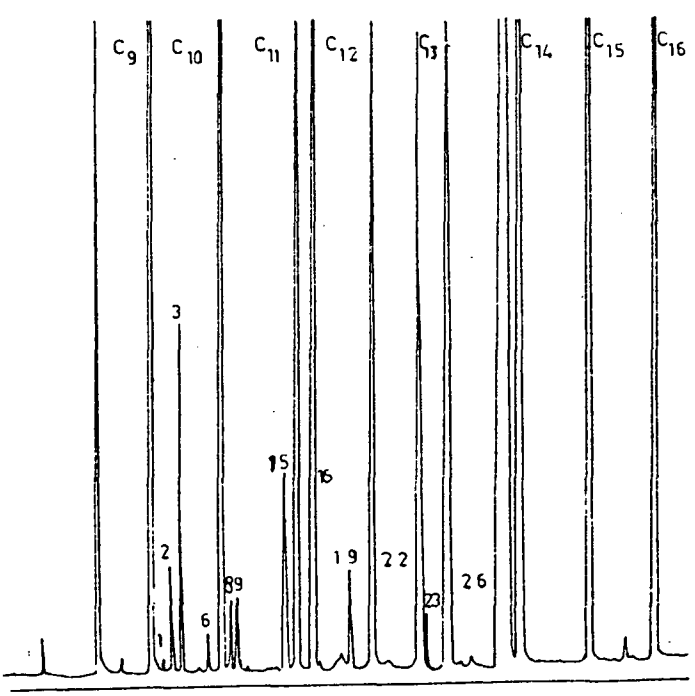

Figure 6. Chromatogram of whole oil of Atherosperma moschatum, OV-101 SCOT column, temperature programmed 50°-200°C at 5°C per minute.

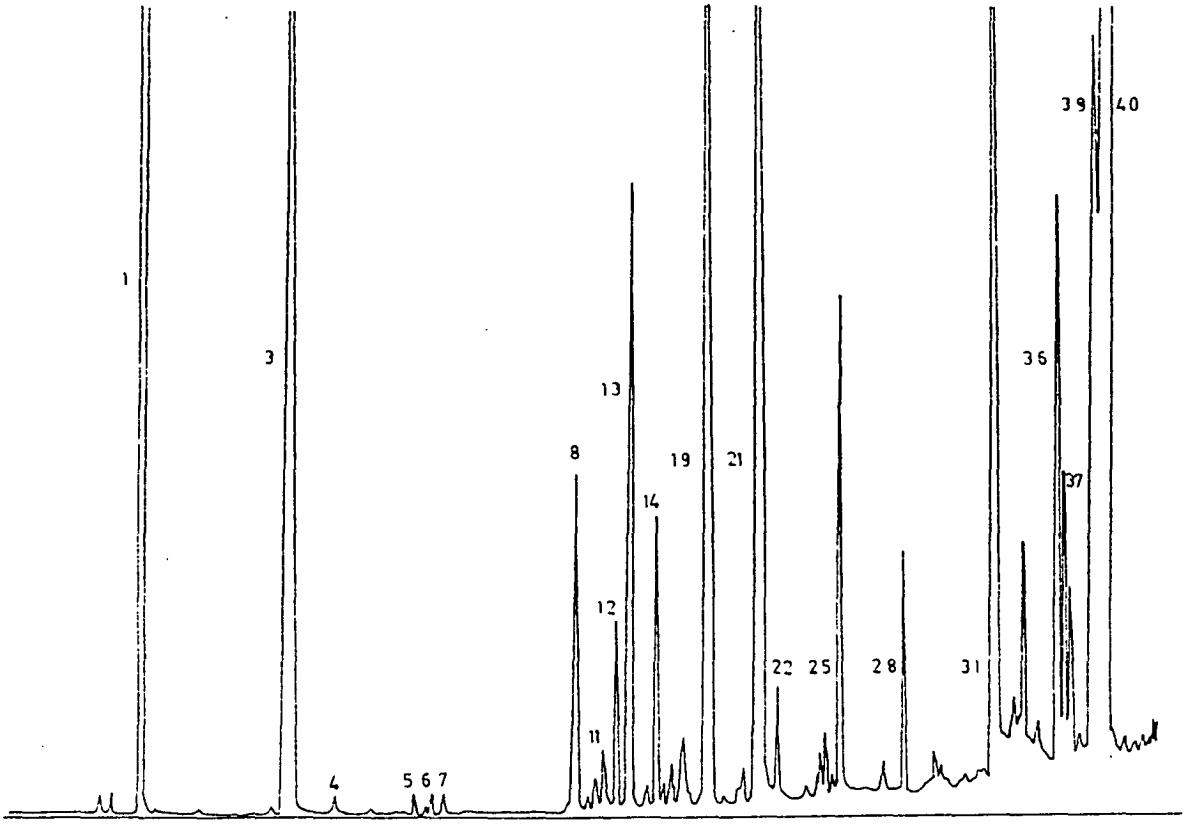

Figure 7. Chromatogram of whole oil of Leptospermum lanigerum CW-20m SCOT column 50°-200°C at 5°C per minute.

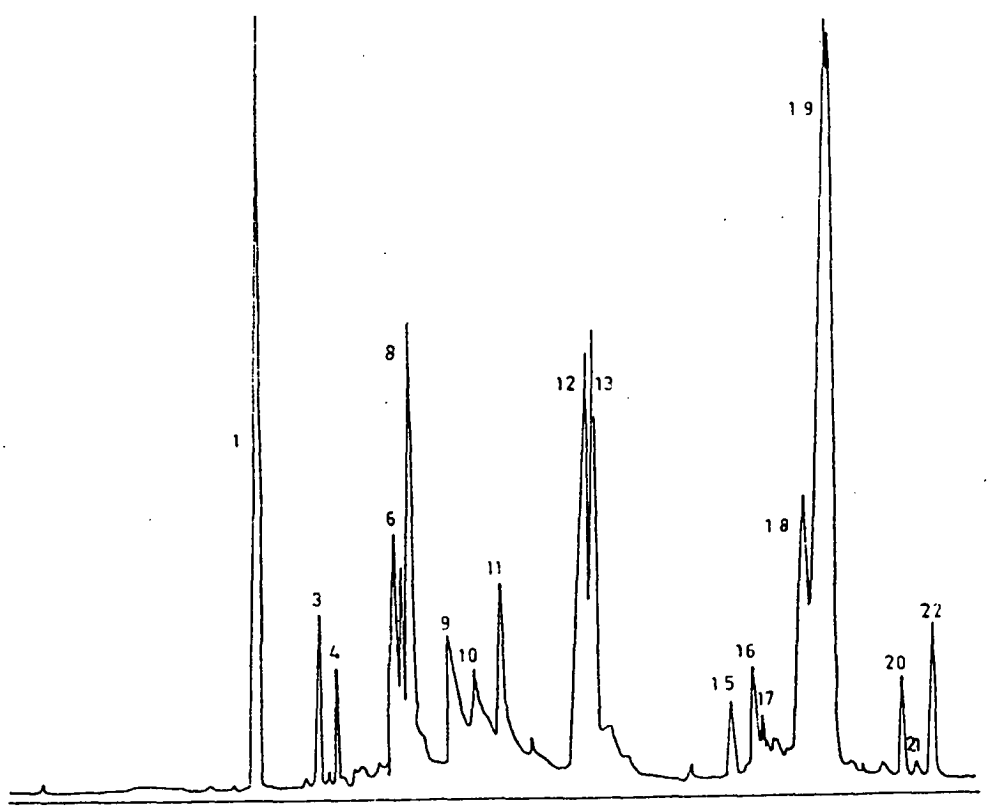

Figure 8. Chromatogram of whole oil of Leptospermum lanigerum OV-101 SCOT column, 50°-200°C at 5°C per minute.

Table 8 Temperature programmed Kovats Indices calculated from Figure 5 for whole oil of A. moschatum, calculated by method of polygonal approximations and spline functions CW-20m SCOT column

Peak No.	I polygonal	I spline	Possible Identification (ref 184)	I	Dual Column Idn.	I
10	997	998	-		α-pinene	998
11	1040	1038	-		camphene	1038
13	1070	1068	-		β-pinene	1068
18	1151	1149	-		myrcene	1149
21	1195	1194	-		α-phellandrene	1177
22	1206	1206	limonene	1206	limonene	1206
23	1216	1216	β-phellandrene	1216	β-phellandrene	1216
26	1228	1228	1,8-cineole	1226	1,8-cineole	1226
36	1448	1487	-		linalool	1506
42	1523	1518	camphor	1518	camphor	1518
43	1655	1657	pulegone	1657	pulegone	1657
44	1698	1698	borneol	1698	borneol	1698
51	1800	1804			safrole	1876
52	1944	1946	-		methyl eugenol	2103

Table 9 Temperature programmed Kovats Indices calculated from Figure 6 for whole oil of *A. moschatum* calculated by method of polygonal approximations and spline functions OV-101 SCOT column

Peak No.	I polygonal	I spline	Possible Identification ref 184	I	Dual Column RRT Idn.
2	938	940	α-pinene	942	α-pinene
3	941	941	camphene	954	camphene
4	949	950	-		-
5	977	978	β-pinene	981	β-pinene
6	988	990	myrcene	986	myrcene
7	994	996	Δ3-carene or α-phellandrene	1002	Δ3-carene or α-phellandrene
8	1069	1010	limonene	1030	limonene
9	1018	1020	p-cymene	1020	p-cymene
10	1023	1023	β-phellandrene	1025	β-phellandrene
11	1033	1034	1,8-cineole	1027	1,8-cineole
12	1058	1060	γ-terpinene	1057	-
13	1064	1064	cis-linalool oxide	1068	-
14	1074	1074	fenchone	1080	-
15	1083	1083	linalool	1087	linalool
16	1121	1122	dihydro linalool	1132	camphor
17	1128	1128	-		-
18	1157	1157	isoborneol	1157	borneol
19	1169	1170	borneol	1164	-
20	1209	1210	carveol	1209	pulegone
21	1219	1220	nerol	1218	-
22	1263	1263	decanol	1263	safrole
23	1266	1267	anethole	1270	bornyl acetate
24	1314	1314	-		-
25	1328	1329	terpinyl acetate	1335	methyl eugenol
26	1382	1382	iso eugenol	1394	-

Table 10 Compounds identified in whole oil of A. moschatum by Kovats Indices and retention data.

Compound	% Composition
α-pinene	1
camphene	3
β-pinene	0.1
myrcene	0.5
α-phellandrene	0.5
Δ3-carene	0.5
β-phellandrene	0.5
limonene	0.5
p-cymene	tr
1,8-cineole	0.1
linalool	4
camphor	10
pulegone	tr
borneol	tr
safrole	12
bornyl acetate	0.5
methyl eugenol	53

literature values (184) are listed. In comparison to those from the CW-20M phase, these indices show closer agreement with literature values for components previously identified by relative retention times, the major deviations being limonene +20 units, pulegone +21 units, safrole +15 units and methy l eugenol -100 units.

Compounds identified and tentatively identified by the combination of dual column relative retention indices and Kovats Indices are listed in Table 10. Additional spectral data are needed in order to make correct assignments to compounds such as methyl eugenol, bornyl acetate and limonene that exhibit anomolous behaviour.

3.4.3.2 Temperature programmed Kovats Indices for identification of components in essential oil of Leptospermum lanigerum

Indices were calculated for compounds eluting on carbowax 20M phase and were assigned tentative identifications as listed in Table 10. Due to the previously found anomalous behaviour of this particular phase, only peaks eluting between index values of 1200 and 1800 were listed. Of the forty compounds eluted, 20 were able to be correlated with available data.

From this phase a number of high temperature compounds were found to elute. These accounted for approximately 30% of the oils' composition. No literature correlations were found for this group of compounds.

Fewer compounds were separated and eluted on OV-101 phase than with CW-20M, and these are assigned tentative identification in Table 11. With this phase the need for optimum separation efficiency in an analytical GC system is demonstrated in that some peaks may contain multiple compounds. For example, Peak No.1, Table 12 could possibly, from examination of components eluted on CW-20M, Table 11, contain limonene, cineole and p-cymene. More spectrometric data are

therefore necessary in order to resolve such discrepancies.

As found with CW-20M phase high temperature components were present in similar concentrations eluting on OV-101. No literature Index values were found corresponding to these compounds. Further spectrometric data are therefore required for identification.

From the comparison of data from components eluting on both these columns, compounds identified in this oil are listed in Table 13 together with their percentage composition.

3.4.3.3 Calculation of Indices by Spline Function

Kovats Indices wre calculated for all compounds eluting on both CW-20M and OV-101 by the method of cubic splines. These are also listed in Tables 8, 9, 11 and 12. The difference in values between Indices calculated by this method and by the polynomial approximation was found to be within two units and therefore within interlaboratory variation. It was concluded that the calculation of Indices by the method of cubic splines, although proposed as being a more accurate approximation (186) did not have any influence on the tentative identification of eluted components by Retention Indices.

3.4.4 Conclusions from Sections 3.2, 3.3, 3.4

The combination of RRT and Kovats Indices calculated for the two essential oils investigated enabled approximately 50% of the compounds separated by GC to be identified with a degree of certainty. Components tentatively identified and requiring further confirmatory spectrometric data were also noted.

The advantages of temperature programmed calculation of Indices over Isothermal calculation was demonstrated for complex mixtures of essential oils. As both oils contained compounds with a wide range of volatilities, temperature programming overcame the large number of tedious calculations necessary for the isothermal method.

Table 11 Temperature programmed Kovats Indices calculated from Figure 7 for the whole oil of L. lanigerum (CW-20M SCOT column) by method of polygonial approximations and spline functions.

Peak No.	I poly-gonial	I spline	Possible Identification	I (Ref.184)	% Composition
2	1204	1205	limonene	(1206)	0.05
3	1228	1-28	1,8-cineole	(1228)	10.7
4	1276	1277	p-cymene	1272	0.11
5	1360	1360	hexanal	1368	0.12
6	1382	1382	nononal	1382	0.14
7	1397	1397	tetrahydrolinalool	1397	0.10
8	1548	1547	linalool	(1506)	1.65
9	1559	1559	?		0.04
10	1565	1564	?		0.26
11	1576	1576	isopulegol	1573	0.43
12	1591	1591	myrcenol	1585	0.38
13	1618	1620	terpinene-4ol	1628	3.61
14	1640	1640	citronellyl acetate	1645	1.43
15	1646	1646	citronellyl acetate	1645	0.06
16	1656	1657	Δ-terpineol	1655	0.22
17	1661	1662	α-terpineol	1661	0.11
18	1674	1674			0.61
19	1707	1707	citronellol	1722	11.9
20	1745	1745	geranyl acetate	1735	0.25
21	1766	1766	?		11.2
22	1778	1778	phenylethyl acetate	1785	0.2

For peaks 23-40, Index values were not calculated as little reliable correlation was available for these values on this phase.

Table 12 Temperature programmed Kovats Indices calculated from Figure 8 for oil of L. lanigerum, OV-101 SCOT column by polygonal method and cubic spline functions.

Peak No.	I polygonial	I spline	Possible Identification	I (Ref.184)	% Composition
1	1024	1024	1,8-cineole	(1027)	10
2	1074	1073	nonanone	(1074)	0.1
3	1087	1086	tetrahydrolinalool	(1086)	1.5
4	1105	1104	myrcenol	(1103)	1.2
5	1129	1128	?		0.6
6	1148	1149	isopulegol	(1145)	3.4
7	1164	1163	terpinen-4-ol or Δ-terpineol	(1175) (1160)	
8	1182	1183	α-terpineol	(1185)	10.5
9	1221	1220	nerol citronellol	(1218) (1215)	5.0
10	1247	1246	linalyl acetate	(1246)	4.1
11	1275	1277	bornyl acetate	(1279)	4.4
12	1370	1372	methyl cinnamate geranyl acetate	(1365) (1364)	13.0
13	1378	1379	unknown		
14	1401	1401	tetradecane	(1400)	0.2
15	1532	1532	unknown		0.9
16	1558	1558			2.7
17	1583	1583			1.0
18	1620	1621	possibly high boiling hydroxylic compounds		7.9
19	1652	1653			24
20	1746	1747			1.0
21	1760	1761			0.2
22	1784	1785			1.8

Table 13 Compounds identified in whole oil of Leptospermum lanigerum by temperature programmed Kovats Indices.

Indice CW-20M	Indice OV-101	Compound	% Composition
1206	1024	limonene	0.1
1228	1024	1,8-cineole	10.7
1277	1024	p-cymene	0.1
1382	1074	nonanal	0.1
1397	1086	tetrahydrolinalool	1.5
1576	1049	isopulegol	0.4
1591	1103	myrcenol	1.2
1620	1175	terpinen-4-ol	3.5
1657	1160	Δ-terpinol	0.2
1662	1185	α-terpineol	10.5
1745	1364	gernayl acetate	13
1707	1215	citronellol	5.0

Reproducibility of Indices within this laboratory was found to be within ±2 units. Although some compounds tentatively identified did not agree with literature values, especially for CW-20M phase, Kovats Indices were found to be of value as a preliminary screening technique for many compounds present in these oils.

Calculation of temperature programmed Indices by the method of cubic spline functions resulted in no variations that were outside experimental error. It was, however, found to be a technique providing rapid interpolation of data in comparison to the polygonal approximation.

It has been demonstrated that ancillary spectrometric data are

required for compounds tentatively identified in both oils in order to give a more positive structural assignment. It has also been demonstrated that further investigation of the high temperature eluting compounds in L. lanigerum and the lower and higher eluting fraction from A. moschatum is needed.

CHAPTER 4

4. Gas Chromatography-Mass Spectrometry for the Identification of components in the oil of L. lanigerum and A. moschatum

4.1 Introduction

Anomalies found in the retention methods of identification of terpenes present in essential oils demonstrate the need for supplementary information in order to confirm identification.

In the analysis of essential oils, the use of GC-MS-computer systems has been a most significant advance in the reliable identification of individual components.

Electron impact spectra have been widely used for the identification of terpenes and spectra have been listed (187). These have provided information complementary to retention data for the identification of many compounds present in essential oils (39).

Certain terpenoids have, however, been shown to exhibit similar retention characteristics together with similar spectra (39). Where this is the case, other spectroscopic data, different ionization techniques, or chemical reaction methods may be necessary to give the correct identification of components.

4.2 Experimental

A VG MM 7070F mass spectrometer equipped with a PYE 204 GC via an open split coupling was used for all GC-MS experiments. The GC conditions were the same as those previously used for retention index measurements.

Samples were injected onto a 50m x 0.2mm silica WCOT column containing the same phases as previously used for the Kovats Indices experiments. A split ratio of approximately 1:10 was used.

Data acquisition was started at the time of injection and mass spectra were recorded every 2 seconds per decade on a VG 223S system coupled to a PDP8/A620 computer. Total ion current chromatograms and extracted ion current profiles were reconstructed by the computer.

The system was operated at 70eV at a vacuum of 4×10^{-6} Torr and the electron impact mode was used for all GC-MS runs. The instrument was calibrated by injection of fluorinated kerosene mixtures, and helium at a flow rate of 1-1.5 ml per minute was used as carrier gas.

Although both GC columns were silica WCOT and not the SCOT columns previously used, a direct correlation of peaks with previous data was able to be made.

4.3 Results and Discussion

4.3.1 Atherosperma moschatum oil

EI spectra were recorded for compounds eluting on GC columns containing CW-20M and OV-101 phases. The total ion current traces are shown in Figures 9 and 10. Identification of corresponding peaks by MS library search are listed in Table 14 for CW-20M phase and Table 15 for OV-101 phase. Identifications found by Kovats Indices and RRT are also given in these tables.

Mass spectra corresponding to Figures 9 and 10 are listed in Appendices 2 and 3 respectively.

MS identification of compounds eluting on CW-20M phase (Table 14)

The anomalous behaviour of carbowax 20M phase found in previous retention index measurements was confirmed with the positive identification by MS of α-pinene (scan 237), camphene (scan 249), and β-pinene (scan 275).

Differences in identification by MS were found for myrcene (scan 156), limonene (scan 179), cineole (scan 191) and p-cymene (scan 231). Identification of these peaks by retention data on this phase was Δ3-carene or α-phellandrene, myrcene, unknown, limonene or β-phellandrene respectively. It has been suggested (142) that rearrangements of terpenes can occur in GC and MS systems. This possibility was found not to be the case as when known compounds underwent GC-MS, they were found to give the correct spectra at the correct retention time. It was therefore found that previous identifications by RRT and KI for these compounds on this phase were anomolous.

Tentative identification by retention data showed that linalool and camphor eluted in the above order. However, MS characterization resulted in the identification being reversed. The elution order was not reversed, since the concentration of each component eluting remained the same for both methods of identification.

Scan 620 confirmed that α-terpineol and not pulegone was present, whilst safrole and methyleugenol were confirmed by their MS, although their retention data did not agree.

MS identification of compounds eluting on OV-101 phase (Table 15)

Better agreement between MS identification and retention data was found for this phase. The only anomolies being p-cymene (scan 318) and limonene (scan 327). Identification was reversed on this column.

MS scan 327 was found also to contain 1,8-cineole as well as limonene. These two compounds were resolved on CW-20M phase.

Conclusions for Atherosperma moschatum

A comparison of MS and retention data on dual columns has enabled the identification of the compounds listed in Table 16.

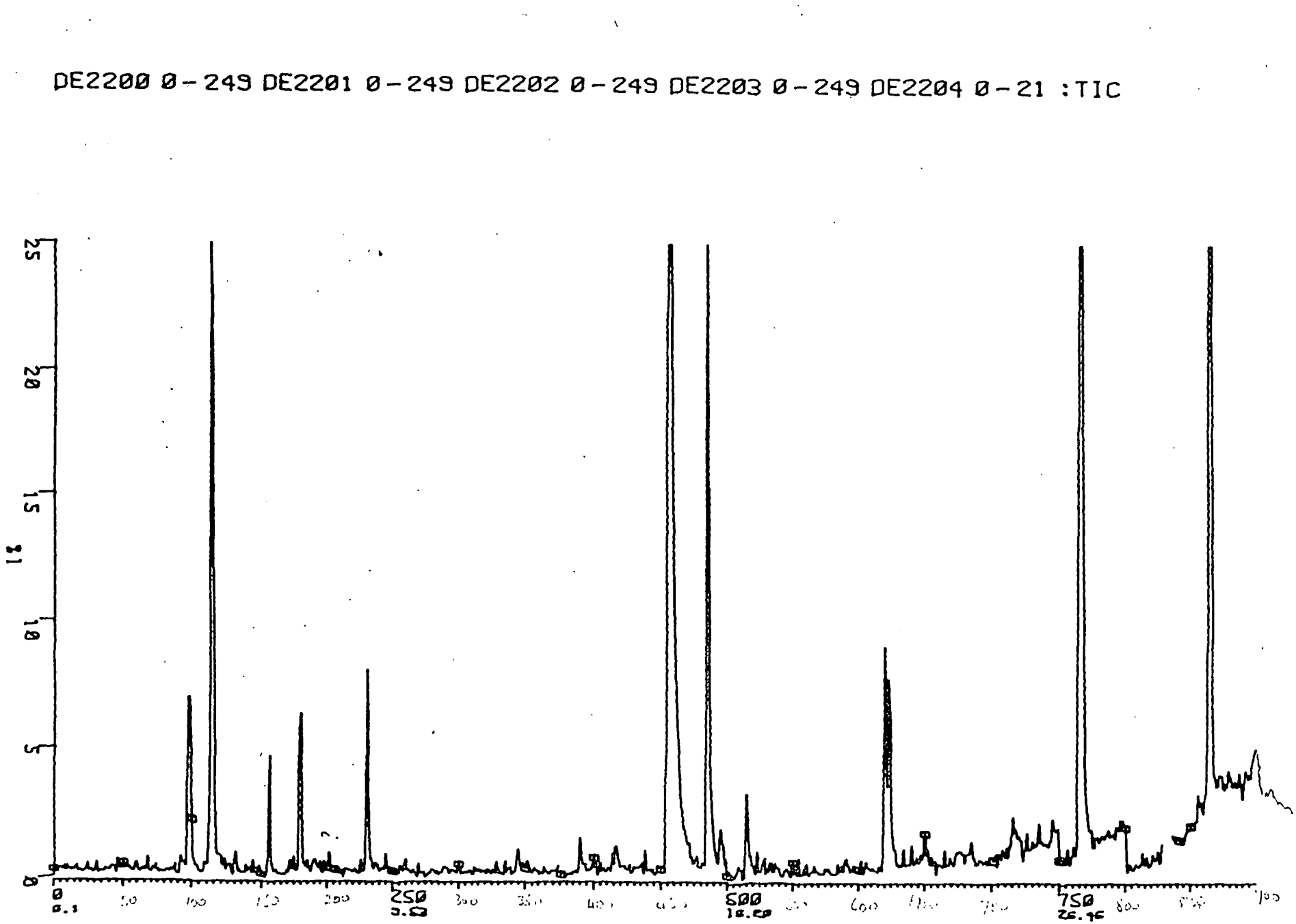

Figure 9. Total ion current trace of whole oil of Atherosperma moschatum, CW-20m silica column temperature programmed 50°-220°C at 4°C per minute.

Table 14 Comparison of compounds isolated by GC and Identified by Techniques of MS, RRT and TPKI for CW-20M phase for *Atherosperma moschatum*.

MS Scan No. Fig.	Identification Technique MS	Kovats Indices	RRT
97	α-pinene		α-pinene
114	camphene		camphene
131	β-pinene		β-pinene
156	myrcene	?	Δ3-carene α-phellandrene
179	limonene	myrcene	myrcene
191	cineole	?	?
202	monoterpene OH	?	?
231	p-cymene	limonene	β-phellandrene
344	fenchone	cineole	?
390	?	?	?
417	?	?	?
455	camphor	linalool	linalool
485	linalool	camphor	camphor
495	?	?	?
15	anethole/estragole	?	?
620	terpinen-4-ol	pulegone	pulegone terpinen-4-ol
623	borneol	borneol	borneol
716	?		?
763	safrole		safrole
856	?		?
863	methyl eugenol		methyl eugenol
932	eugenol		
939	methyl eugenol isomer		

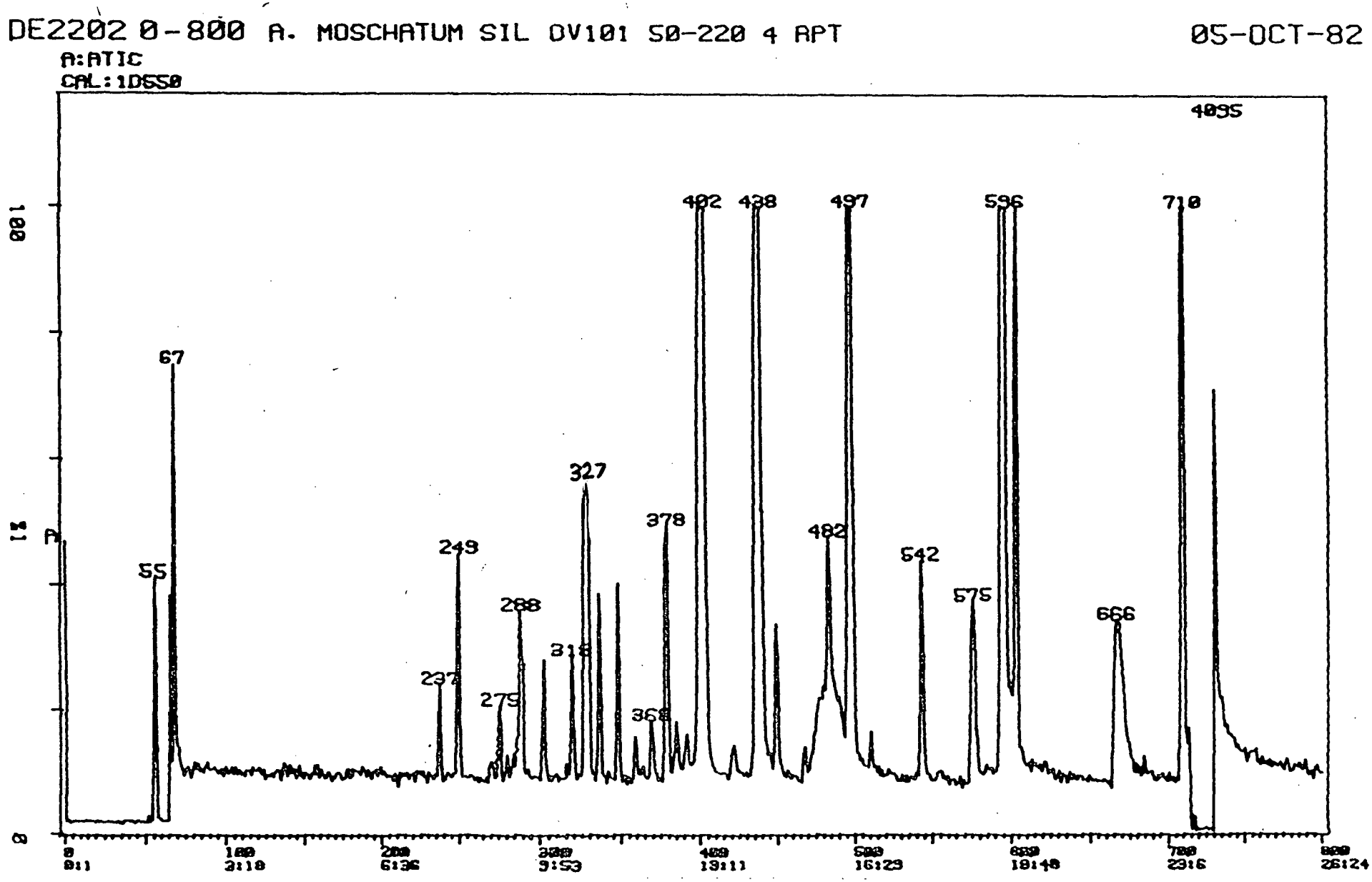

Figure 10. Total ion current trace of whole oil of Atherosperma moschatum, OV-101 silica column, temperature programmed 50°-220°C at 4°C per minute.

Table 15 Comparison of compounds isolated by GC from whole oil of *A. moschatum* and identified by techniques of MS, RRT and TPKI (OV-101 phase).

	Identification Technique - Temperature Programmed		
MS Scan No. Fig.	MS	Kovats I	RRT
237	α-pinene	α-pinene	α-pinene
249	camphene	camphene	camphene
275	β-pinene	β-pinene	β-pinene
288	myrcene	myrcene	myrcene
302	α-phellandrene	Δ3-carene/ α-phellandrene	
318	p-cymene	limonene	limonene
327	{ 1,8-cineole limonene	cineole, p-cymene β-phellandrene	
335	trans-ocimene		
347	β-ocimene		
358	monoterpene		
368	monoterpene OH		
378	fenchone		
402	linalool	linalool	linalool
421	? unknown		
438	camphor	camphor	camphor
448	unknown		
482	terpinen-4-ol		
497	borneol	borneol	nerol/borneol
	anethole/estragole		
510	unknown		
542	unknown		
575	unknown		
596	safrole	safrole	safrole
606	bornyl acetate	bornyl acetate	bornyl acetate
666	eugenol		
710	methyl eugenol	methyl eugenol	methyleugenol

Table 16 Compounds present in whole oil of Athersperma moschatum by GC-MS on dual columns.

Scan No. OV-101	Scan No. CW-20M	Compound	% Composition
237	97	α-pinene	1.0
249	114	camphene	3.0
275	131	β-pinene	0.1
288	156	myrcene	0.6
327	179	limonene	0.9
327	191	1,8-cineole	0.2
368	202	monoterpene alcohol	0.1
318	231	p-cymene	0.8
302		α-phellandrene	0.7
335		trans-ocimene	0.9
347		β-ocimene	1.0
358		monoterpene	<0.1
378	344	fenchone	2.4
	390	unknown	<0.1
	417	unknown	<0.1
384		unknown	<0.1
438	455	camphor	3.0
402	485	linalool	6.0
421		unknown	<0.1
	495	unknown	<0.1
448		unknown	1.1
482	620	terpinen-4-ol	2.4
497	623	borneol	2.8
497	515	anethole	0.2
} onside		{	}
497	515	estragole	
510		unknown	<0.1

Table 16 continued:

542		unknown	1.0
575		unknown	1.8
	856	unknown	0.4
596	763	safrole	10.7
606		bornyl acetate	4.2
666	932	eugenol	2.1
710	939	methyl eugenol	51

Unknown compounds were found in low concentrations and either clear spectra were not available or the spectrum was not available in the library.

4.3.2 Leptospermum lanigerum oil

EI spectra were recorded for compounds eluting on GC columns containing CW-20M and OV-101 phases. The total ion current traces are shown in Figures 11 and 12. Identifications of corresponding peaks by MS library search are listed in Tables 17 for CW-20M phase and Tables 18 for OV-101 phase. Identifications found by Kovats Indices and retention data are also given in these tables.

Mass spectra corresponding to Figures 11 and 12 are listed in Appendices 4 and 5 respectively.

MS Identification of compounds eluting on CW-20M phase (Table 17)

As in previous experiments with this phase, agreement between mass spectra of compounds eluted and Kovats Indices between 1200 and 1800 were found, no correlations wre attempted outside this range.

The greater resolving power of WCOT columns in comparison to SCOT columns was demonstrated in that geranyl acetate (scan 584) and citronellol (scan 596) were resolved. These compounds eluted as a

mixture on SCOT columns of this phase.

Mass spectral correlations were not found for compounds eluting between scan 861 and scan 994. (No retention index data correlations were previously found for these compounds). Their mass spectra suggested that they were predominantly sesquiterpenes with an alcohol group present. This confirmed previous IR studies by Ayling (176) on the crystalline material separated from L. lanigerum. Flynn et al (188) in their work on similar Leptospermum species reported similar compounds and identified them as eudesmols.

MS Identification of compounds eluting on OV-101 phase (Table 18)

General agreement with Kovats Indices and Mass spectra of compounds eluting on this phase was found, and several previously tentatively identified compounds were able to be confirmed.

The same high boiling sesquiterpene compounds were found in approximately the same concentration, although on this phase the elution order of the two major components was reversed, i.e. scan 964 and scan 971 correspond to scan 990 and scan 982 respectively on the CW-20M phase (Table 17).

Conclusions for L. lanigerum

The comparison of Kovats Indices and MS data for components in this oil enabled the identification of compounds as listed in Table 19.

Some major components were not able to be identified by MS as no data was available for specific compounds. An interpretation of these spectra enabled tentative assignment as to the class of compound.

Trace components were not identified by MS due to poor spectra at the detection limit.

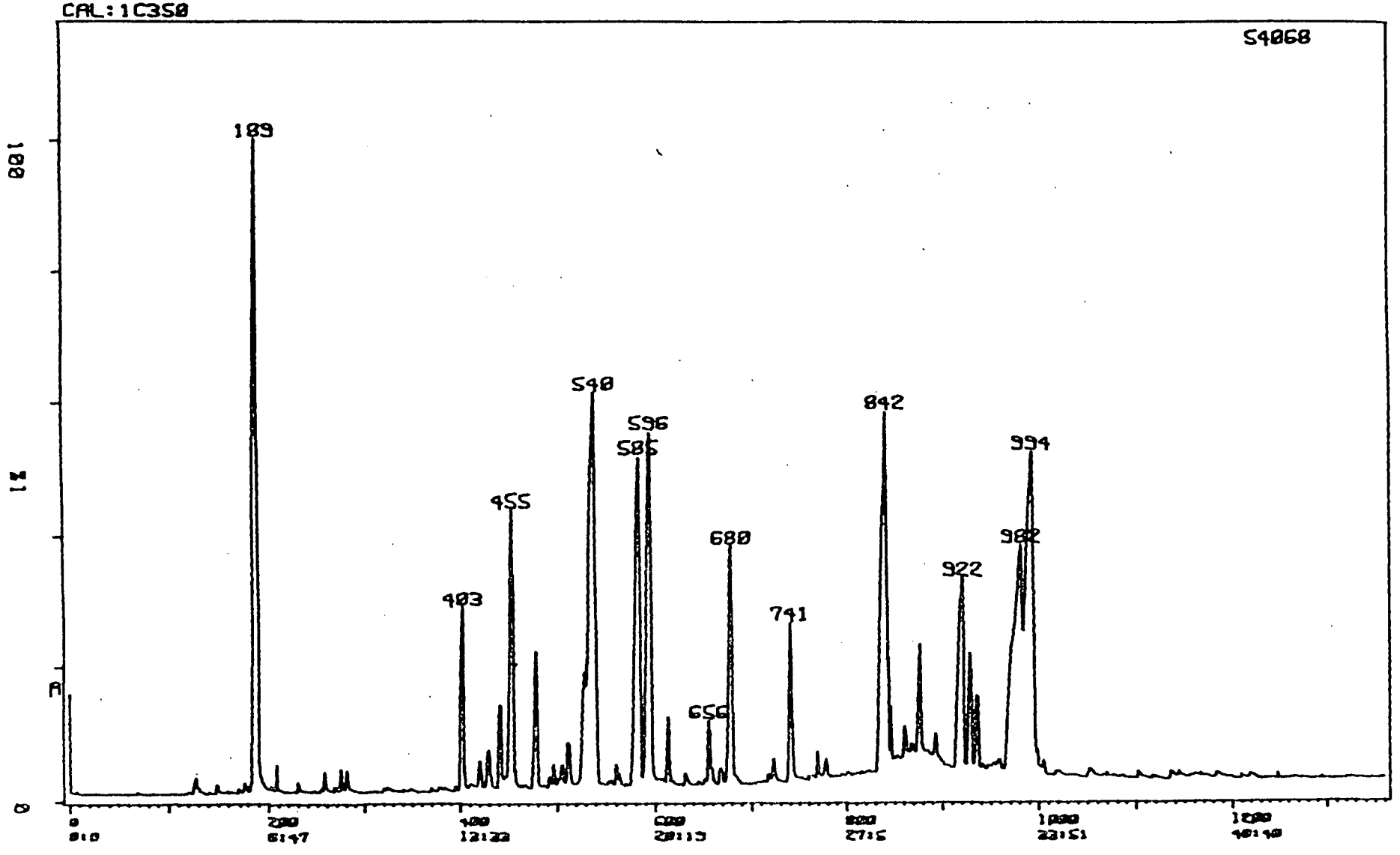

Figure 11. Total ion current trace of whole oil of L. lanigerum CW-20m silica column, temperature programmed 80°-200°C at 4°C per minute.

Table 17 Comparison of identification technique of compounds isolated by GC from oil of Leptospermum lanigerum on CW-20M phase.

Scan No.	Identification Technique MS (Figure 11)	Kovats Indices
149	α-pinene	-
176	β-pinene	limonene
185	1,8-cineole	1,8-cineole
208	me-isopropyl benzene	p-cymene
258	?	hexanal
275	hexenol	nonanal
281	?	tetrahydrolinalool
403	linalool	linalool
442	?	isopulegol
455	terpinene 4-ol	terpinene 4-ol
479	myrtenal	citronellyl acetate
505	citronellyl acetate	citronellyl acetate
534	terpineol	-terpineol
584	geranyl acetate }	citronellol +
596	citronellol	geranylacetate
614	myrtenol	?
656	phenyl ethyl acetate	phenyl ethyl acetate
680	linalyl acetate	?
741	me cinnamate + unknown	
836	me cinnamate isomer	
861	sesquiterpene alcohol	
877	?	
922	?	
929	?	
937	sesquiterpene	
970	?	
982	?	
994	?	

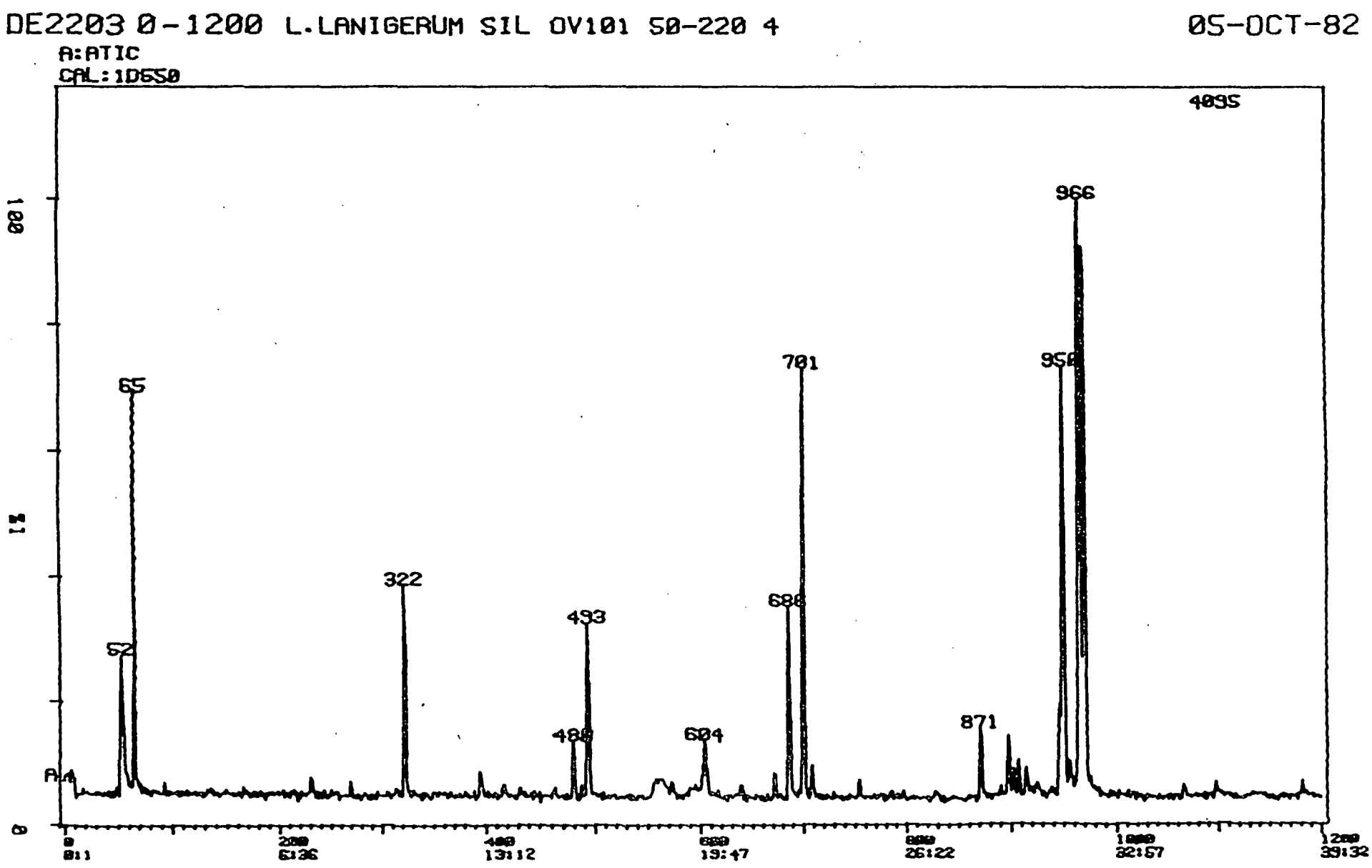

Figure 12. Total ion current trace of whole oil of L. lanigerum OV-101 silica column, temperature programmed 50°-220°C at 4°C per minute.

Table 18 Comparison of identification techniques of compounds isolated by GC from oil of Leptospermum lanigerum on OV-101 phase

Scan No.	Identification Technique MS (Figure 12)	Kovats Indices
232	α-pinene	tr
270	β-pinene	tr
322	1,8-cineole	1,8-cineole
396	linalool	tetrahydrolinalool
420	? tr	tr
442	? tr	tr
488	terpinen-4-ol	Δ terpineol or terpinen 4-ol
493	α-terpineol	α-terpineol
559	? tr	nerol/cintronellol
573	? tr	linalyl acetate
602	methyl cinnamate	
604	unknown	bornyl acetate
607	2-decanone	
640	?	?
673	citronellyl acetate	geranylacetate ?
686	methyl cinnamate isomer	methyl cinnamate
701	geranyl acetate ?	geranyl acetate ?
857	?	
898	?	
903	?	no correlation
908	sesquiterpene	with data
915	?	available
920	?	
926	sesquiterpene OH	
940	?	
951 ↓ 1182	unknown compounds	unknown compounds

Table 19 Compounds found present in whole oil of Leptospermum lanigerum by dual column GC-MS

Scan No. OV-101	Scan No. CW-20M	Compound	% comp.
52		ethanol } solvent	-
65		chloroform	-
232	140	α-pinene	tr
270	176	β-pinene	tr
322	185	1,8-cineole	10
	208	isopropylbenzene	tr
	258	unknown	tr
	275	hexenol	0.1
	281	unknown	0.1
396	403	linalool	1.5
442	418	unknown	0.2
	433	unknown	0.4
	480	unknown	tr
420		unknown	tr
488	455	terpenen-4-ol	3.5
	479	myrtenol	1.0
673	505	citronellyl acetate	tr
	512	unknown	tr
640	527	unknown	0.1
493	534	α-terpineol	8.1
559		unknown	0.2
573		unknown	tr
602	741	methyl cinnamate	1.2
686	836	methyl cinnamate isomer	8.0
607		2-decanone	tr
604		unknown	tr

Table 19 cont.

701	584	geranyl acetate ?	5.6
	596	citronellol	5.9
	614	myrtenal	0.4
	656	2 phenyl ethyl acetate	0.4
	668	unknown	tr
	680	linalyl acetate	2.7
	723	unknown	tr
872	846	unknown	2.1
946		unknown	tr
857		unknown	tr
898	877	unknown	tr
903		unknown	tr
	861	sesquiterpene alcohol	tr
908	929 937	sesquiterpene	0.8
915		unknown	tr
920		unknown	tr
926		sesquiterpene alcohol	0.7
940		unknown	tr
951	922	possibly	2.1
964	982	sesquiterpene	8
971	990	alcohols	24
1098		similar to	tr
1182		970, 982	tr

4.4 Conclusions

The positive identification of the majority of components present in the two essential oils investigated has been accomplished by a combination of GC-MS and retention data.

Lack of data for some components necessitates the need for further spectrometric and chemical investigation. This has been demonstrated with the high boiling group of compounds found in L. lanigerum tentatively identified as sesquiterpene alcohols.

It has been shown that tabulated retention data must be used with caution and only as an ancillary technique to spectrometric identification.

Problems encountered where peaks were not well resolved and their possible effect on retention data were found. The need for a column giving maximum separation is thus necessary.

The large number of compounds with similar characteristics sometimes made base line separations difficult and thus quantitation from the plotting integrator must be considered approximate.

CHAPTER 5

5. Raman Spectroscopic Identification of Gas Chromatographic Fractions isolated from oil of Atherosperma moschatum

5.1 Introduction

The inability to identify unequivocally some volatile compounds eluting from a gas chromatograph by retention data and mass spectrometry demonstrates the need for further spectral data to assist in structural assignments.

Raman spectroscopy in conjunction with other spectroscopic methods has been long recognised as a technique for the characterization of molecular structures. In conjucntion with IR it can give a more complete picture of the vibrational behaviour of the molecule.

Apart from its use in structural investigations of natural products (189, 190), Raman spectroscopy has found applications in identification by fingerprinting techniques, similar to IR spectral matching.

In the past, sample size requirements have limited the general application of the technique to macroquantities of natural products. However, the use of continuous lasers with their high intensity and low beam divergence as an excitation source, has allowed detection limits of 10 to 100 ng to be obtained. This enables Raman spectroscopy to be used for the identification of collected GC fractions.

Although on-line GC-spectroscopic techniques have advantages

with respect to less contamination, better detection limits and simplicity of operation, off-line GC spectroscopic methods have found wide application where expensive instrumentation is not available.

Off-line collection of GC fractions for subsequent Raman and infrared analysis may be accomplished by trapping the GC effluents in glass capillaries. The co-axial viewing technique allows Raman radiation to be collected from the same tubes and thus obviates the need for sample transfer with attendant losses.

Ayling (150) has reviewed the various GC micro sampling techniques applicable to Raman spectroscopy and they have also been discussed in Chapter 1 of this thesis. Although several workers (167, 168) used these techniques earlier for the identification of terpenes, few recent discussions of the use of off-line GC-Raman spectrometry have been found.

5.2 Experimental

Construction of Effluent Splitter

Few commercial GC effluent splitter attachments are available with low dead volume (so as not to interfere with gas flow geometry) and variable split-ratio capability.

Previous workers have constructed a variety of effluent splitters, the majority of which have a split ratio of 90 or 100 to 1. With such a high ratio, these all require the use of a suitable make up gas in order to prevent adverse effects on the hydrogen flame ionization detector.

A complex system was proposed by Brownlee and Silverstein (191) where make up gas was needed in order to maintain gas flow criteria and peak shapes.

In order to minimize the complexity of these systems, a splitter was constructed for use without make up gas, and having a variable split ratio that did not interfere with the operation of the FID. The instrument used for construction of this attachment was a Tracor 550GC. It was chosen for modification because it affords easy access to the required parts.

The GC column effluent was split by using an SGE capillary column inlet splitter. This device is normally used for the rapid uniform splitting of a mixture of carrier gas and volatile sample before entry into a GC capillary column. The modified splitter is shown in Figure 13.

The splitter was attached in the column effluent line between the packed column and FID and was wrapped with heating tape to maintain a constant operating temperature. The exit port of the splitter was fitted with a 1/16 inch swagelock union containing a graphlock ferrule which accepted the capillary collection tubes. One half turn of the nut ensured a gas tight joint and allowed for the rapid change of capillaries for the collection of successive peaks.

Splitting ratios were adjusted by manipulation of the needle valve and measurement of the gas flow by bubble meter from the

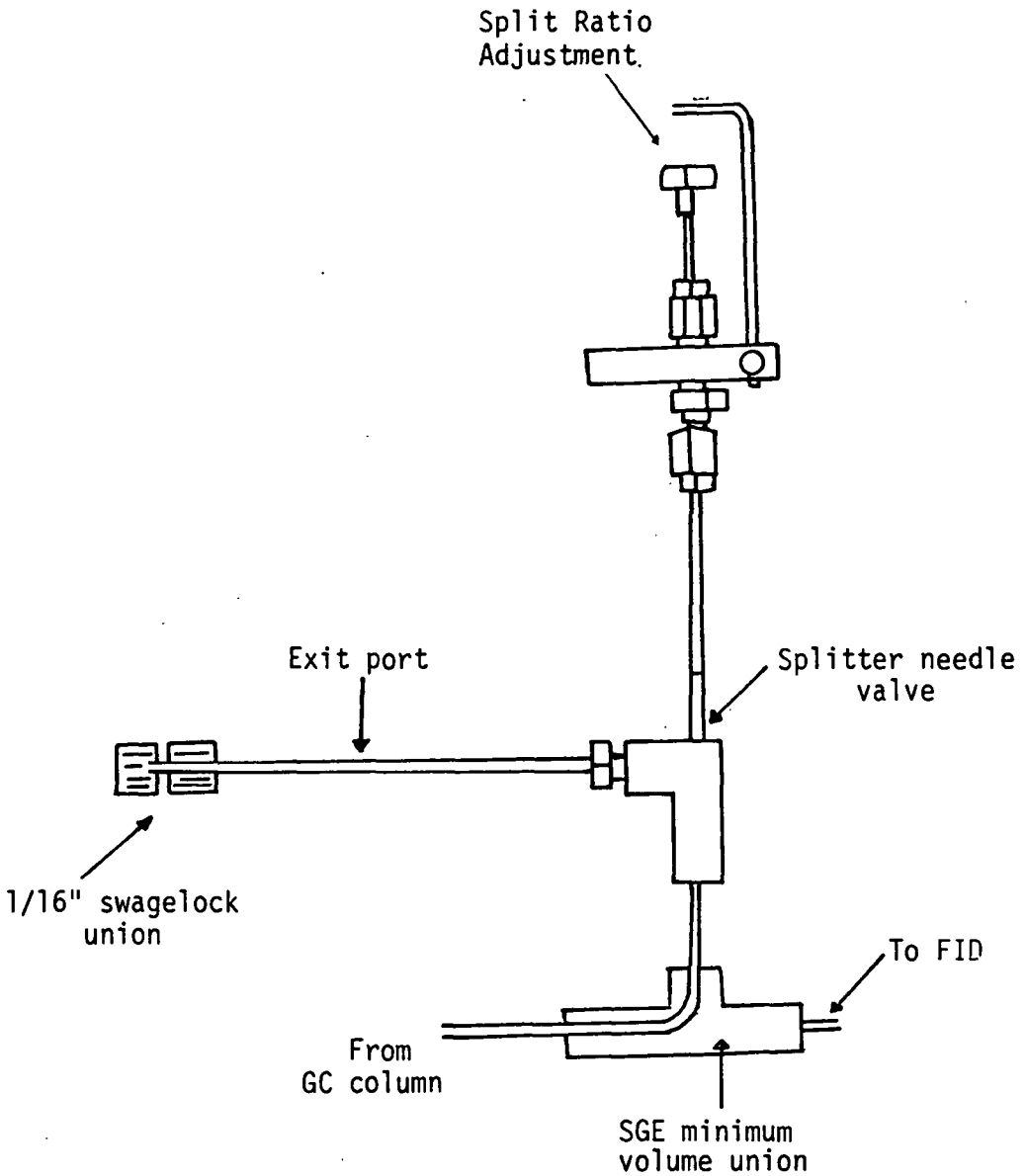

Figure 13. Conversion of SGE Inlet Splitter for use as an effluent splitter.

outlets to the FID and capillary collector. By trial injections of ethanol it was found that a split ratio of approximately 50 to 1 (capillary:FID) gave minimal changes in response of FID and peak shapes.

This system reduces the complexity of sample trapping in that no make up gas is required and yet it still has the ability to operate at a split ratio of 50 to 1.

Construction of a Thermal Gradient Collector

Thin walled glass capillaries have been found to be efficient collectors of GC fractions. Uncooled sections of capillaries are widely used for rapid fraction collection in many laboratories. They do, however, suffer from their inability to collect compounds with a wide range of volatilities. The use of a heated section of capillary has been shown to act as a fog breaker (192), however a device where a temperature gradient may be applied to a capillary tube would appear to be the method of choice for efficient collection of a wide range of compounds (191).

A temperature gradient fraction collector that facilitates handling of 30 cm x 1.6 mm diam. borosilicate glass capillaries was thus constructed (Figure 14).

It consists of a 1.7 mm ID aluminium tube that accepts a 30 cm length of capillary sample collection tube which at one end passes through a 50 cm cube of aluminium containing a 50 watt cartridge heater. At the other end the tube passes through a 50 cm cube aluminium block that has a 25 mm aluminium rod with extends to a dewar flask containing liquid nitrogen. Both blocks were insulated from each other by mounting on an adjustable asbestos platform which has provision for both vertical and lateral alignment of the aluminium tube with the exit port of the GC.

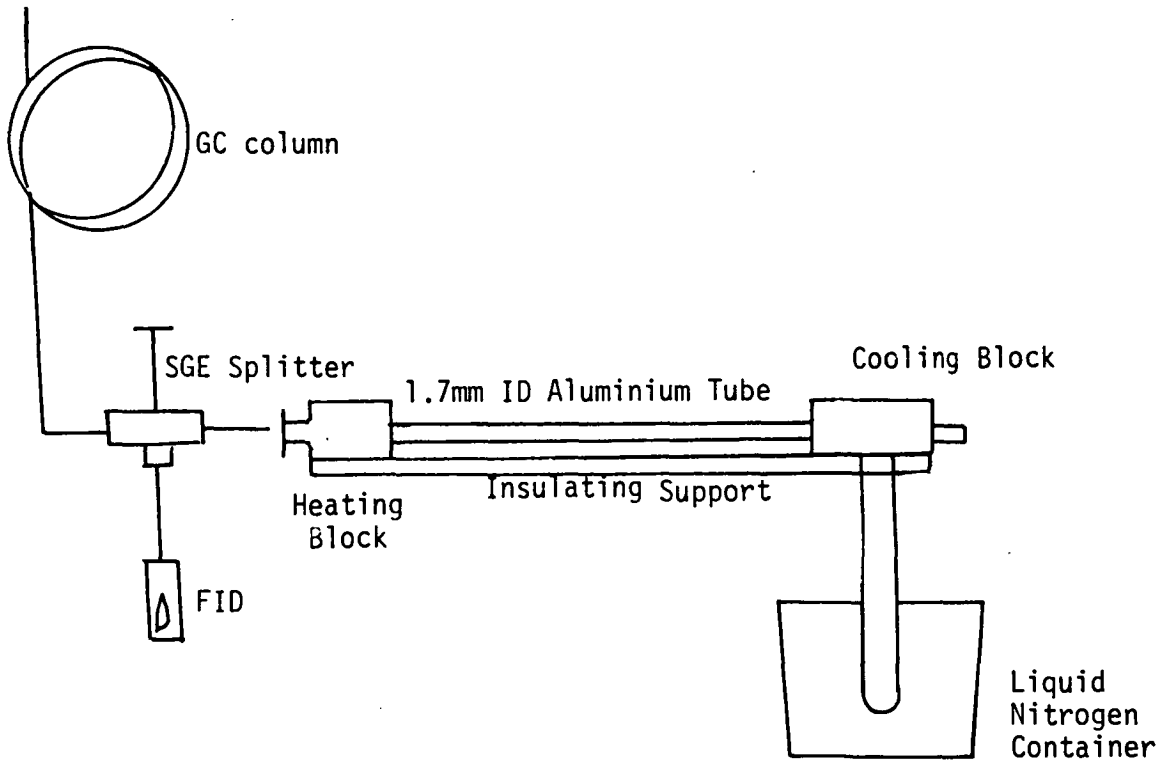

Figure 14. Temperature Gradient capillary cold trap.

The temperature of the heating block was adjusted by a Variac until frosting of the aluminium capillary tube acceptor occurred over approximately half of its length. It was then assumed that an even temperature gradient existed over the mid section of the capillary tube.

Efficiency of the Capillary Collection Device

With any GC fraction collection device it is important to establish the efficiency of such a system. Recoveries of a range of compounds were determined by injection of a 1 µℓ sample of the oil of A. moschatum followed by re-injection of the collected fraction. Comparison of the relevant peak areas of a chromatogram of the collected fractions and that of the FID trace of the split sample

showed that the majority of components had been split and collected quantitatively. The exceptions were α-pinene and camphene which only gave a 72% and 81% recovery respectively, perhaps due to their low boiling point. All other compounds gave recoveries of greater than 90%.

Collection of GC Fractions Isolated from A. moschatum

Aliquots (1.0 μℓ) of the whole oil was injected onto the GC column. GC fractions were collected from the time when a peak of interest began to rise on the recorder trace. Collection was stopped just after the apex of the peak. This time scale was chosen after calculation of the residence times of the compound in the splitter and FID. Pure components were thus trapped and contamination by adjacent peaks was minimized.

With the oil of A. moschatum, the three major compounds comprise approximately 60% of the oil. A "heart cutting" technique was developed whereby after collection of the individual major components, the minor compounds were repeatedly collected as a whole subfraction. This bulked subfraction was then re-injected and the minor components collected. For compounds present in amounts less than 2%, successive injection of this subfraction and collection of compounds was necessary in order to obtain enough material for acceptable spectra.

The FID trace of components collected is shown in Figure 15. Known compounds were further purified by this micropreparative GC technique and their Raman and mass spectra were recorded.

Recording of Raman Spectra

A Carey Model 82 Raman spectrometer, coupled to a Spectra Physics SP 164-08 4 watt Argon laser was used for this investigation.

The 1.6 mm OD borosilicate capillaries containing the collected GC fractions were aligned in a sample holder that allowed transverse viewing and collection of the scattered radiation at 90° to the

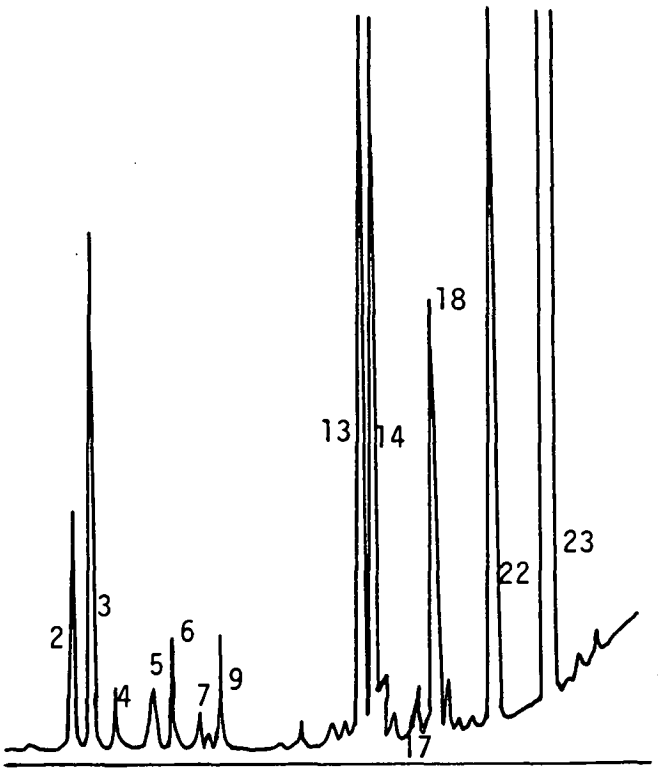

Figure 15. Temperature programmed chromatogram of whole oil of Atherosperma moschatum CW-20m packed column, Temperature programmed 50°-200°C at 5°C per minute With split ratio 50 to one.

incident beam. The ease of recording these spectra contrasts with the difficulties encountered in recording IR spectra of micro quantities with its complex sample handling techniques.

Where extremely small droplets were deposited in the capillary tube, the end was flame sealed and the tube centrifuged in order to collect all the sample in one location. By using this technique, good Raman spectra were able to be obtained on 50 ng of material. The spectra of collected peaks are shown in Appendix 6.

As Raman spectroscopy is a non-destructive technique, compounds collected in these capillaries are able to be studied by other

spectroscopic techniques. Mass spectra were also recorded on all collected fractions for comparison with spectra previously recorded by combined GC-MS.

5.3 Results and Discussion

By using GC fraction collection techniques and Raman spectral viewing techniques, spectra enabling identification of all peaks labelled in Figure 15 were obtained. These spectra are listed in Appendix 6.

The comparison of Raman peak heights showed that the sensitivity of this technique was approximately half of that obtained with a normal 2 ml cell holder. Although this reduction in sample size increased the intensity of spurious peaks and background noise, the Raman intensity appeared only to be slightly reduced.

Compounds present in the oil in high concentrations, i.e. safrole (≅10%), gave a good spectrum after collection of only one peak. This represented a sample weight of approximately 100 ng. The spectrum of peak 22 identified as safrole is shown as an example in Figure 16, together with a spectrum of safrole purified by preparative GC (Figure 17).

For components present in concentrations of approximately one percent, good spectra were obtained for comparison with known pure compounds. For example, Figures 18 and 19 show the comparison of spectra of (-)-limonene isolated from the oil (peak 6) and a spectrum of (-)-limonene purified by GC. Both these spectra represented a sample weight of approximately 50 ng. As the spectrometer was not operating at its maximum sensitivity, an ultimate detection limit of about 10 ng may be expected. This would be comparable with that found by other workers.

Raman spectra for peaks 13 and 14 verified the GC-MS identification

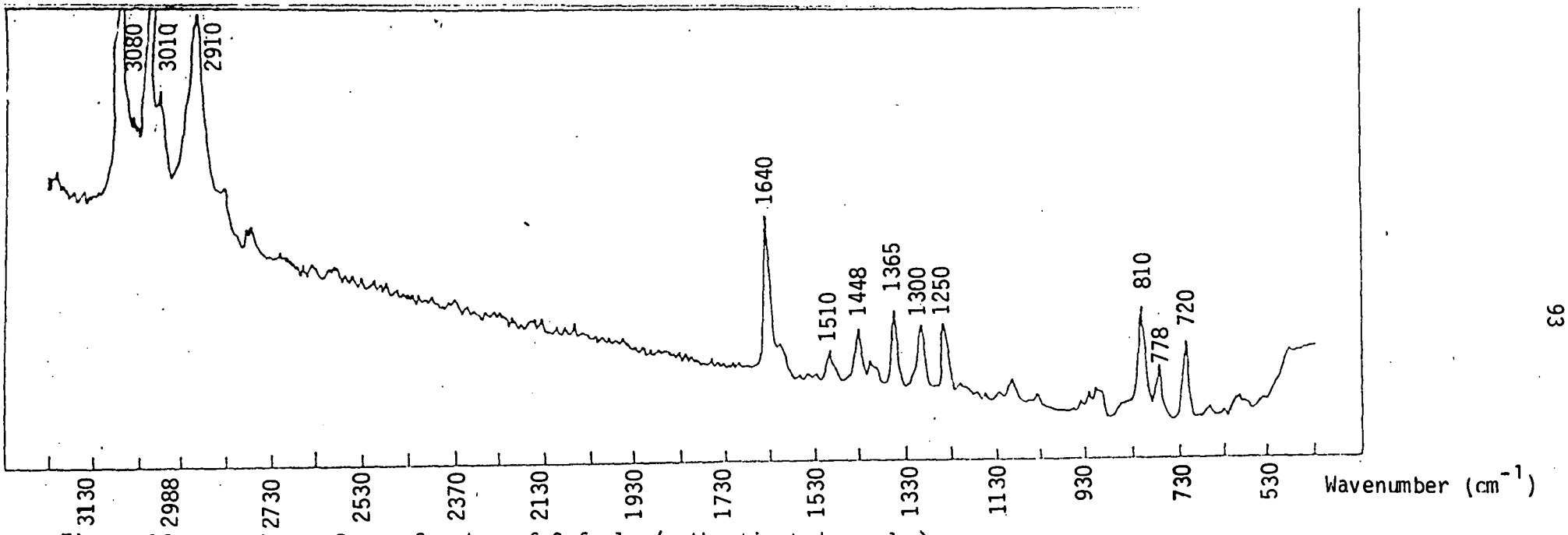

Figure 16. Laser Raman Spectra of Safrole (authenticated sample.)

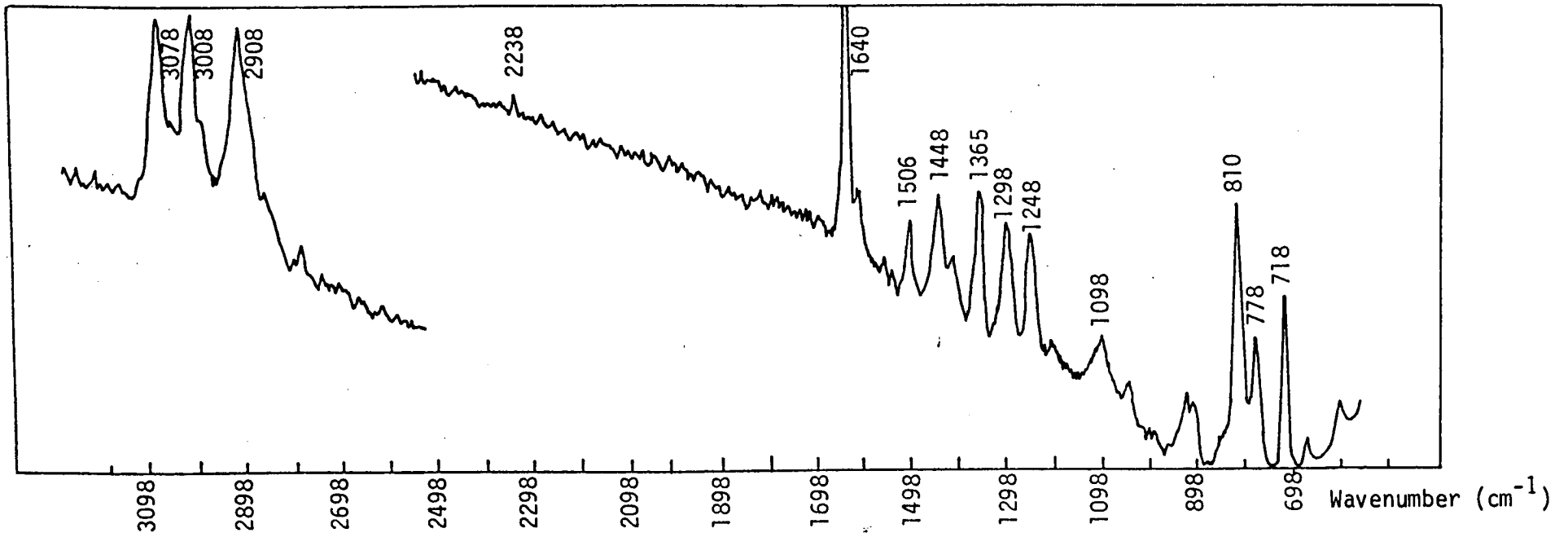

Figure 17. Laser Raman Spectra of Trapped Component No. 22 from A. moschatum. Identified as Safrole.

Sens. 1000 c/s
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PP 20 secs.

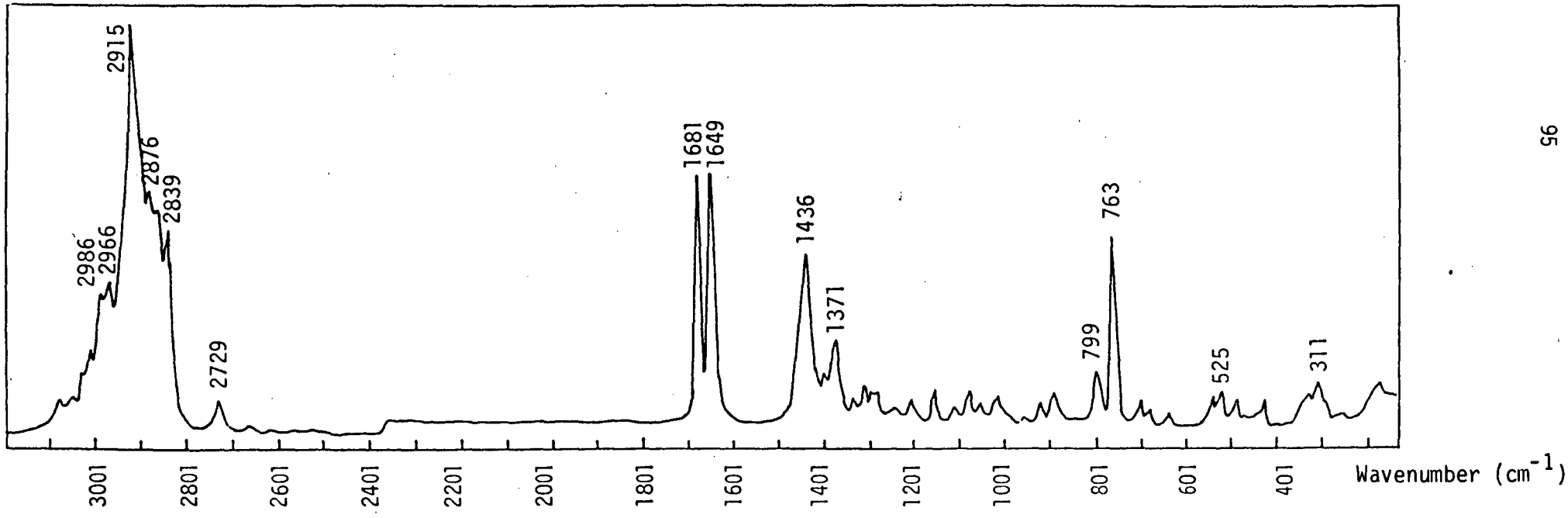


Figure 18. Laser Raman Spectrum of Trapped Component No.6 from Atherosperma moschatum. Identified as (-)-Limonene.

Sens. 2000 c/s
SBW 8.0 cm^{-1}
PP 10 secs.

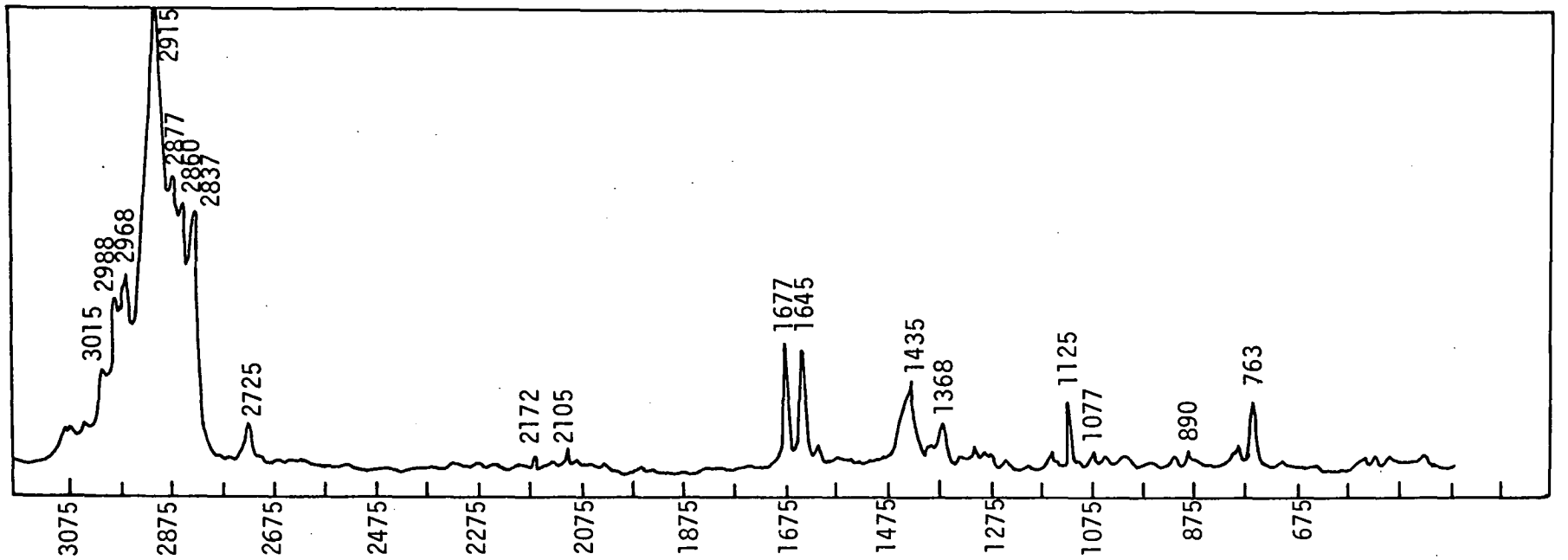


Figure 19. Laser Raman Spectrum of GC purified (-)-Limonene

Sens. 2000 c/s
SBW 4 cm^{-1}
PP 10 secs.

of these two peaks, as camphor and linalool respectively. This confirmed that incorrect identification by retention data had been made for these two compounds.

Probe mass spectra were recorded on all the compounds collected in the capillaries. These were all in close agreement with spectra obtained by combined GC-MS for the corresponding peaks.

5.4 Conclusions

Sampling and analytical techniques for the identification of trace (nanogram) to percentage amounts of constituents present in essential oils by Raman spectroscopy has been accomplished.

Although this off-line technique is not as simple as combined GC-on-line methods, it does fill a need where extra spectral data is required or when expensive on-line instrumentation is not available.

The sampling methodology should prove useful for the collection of micro quantities of volatile compounds for subsequent spectral or micro chemical reaction analysis, especially for the sesquiterpene compounds present in L. lanigerum.

CHAPTER 6

6. Chemical Reaction GC-MS for the Identification of Compounds in Essential Oils

6.1 On-line pre-column hydrogenation GC-MS

6.1.1 Introduction

Vapour phase hydrogenation for the determination of the number of double bonds in a compound is a long established method (193) and its application to GC and GC-MS identification has been previously reviewed in Chapter 1 of this thesis.

Generally post-column hydrogenation is necessary for the analysis of complex mixtures owing to the fact that the vast number of hydrogenated peaks produced are difficult to correlate with non-hydrogenated data.

On-line pre-column hydrogenation is generally applicable to single compounds or simple mixtures. In comparison to off-line techniques, on-line methods generally require less sample, are easier to carry out and are less time consuming.

Varying catalytic conditions have been investigated for both on-line and off-line hydrogenation (103). It has been found that with most terpenes when under a temperature of 175-200°C hydrogenation is effected without hydrogenolysis occurring. The most common catalyst used is palladium on charcoal with hydrogen or a mixture of hydrogen and nitrogen as carrier gas.

In order to obtain structural information to assist in the identification of the major compounds found in L. lanigerum, pre-column hydrogenation GC-MS was investigated as a method for the determination of the number of double bonds present.

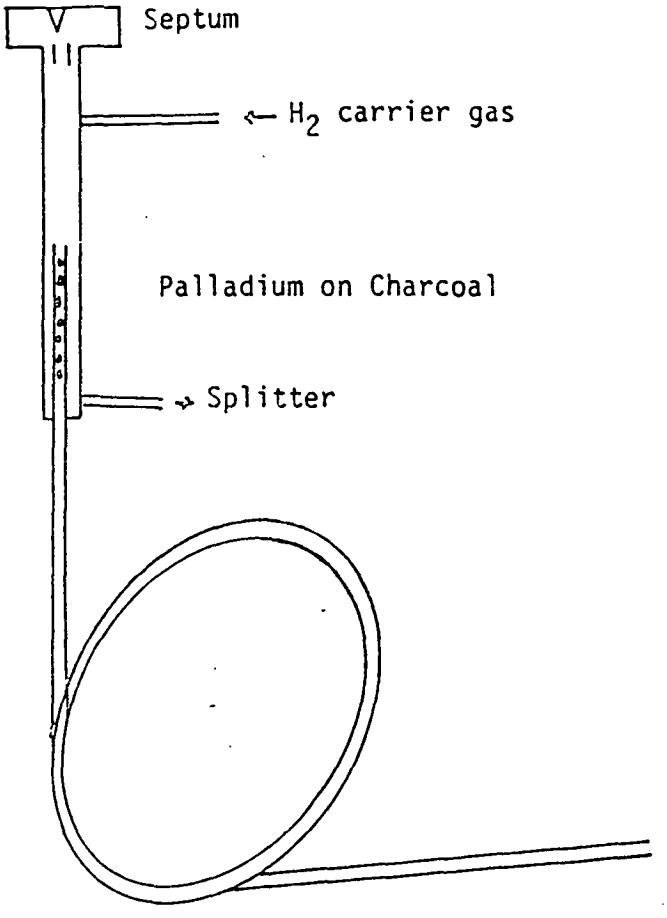


Figure 20. On-Column Hydrogenator

6.1.2 Experimental

Construction of a pre-column hydrogenator

The most commonly used apparatus is modelled on the Beroza Carbon Skeleton Determinator (196) which connects to the front end of a GC column via a syringe needle. This type of apparatus is only applicable to packed columns.

An on-line pre-column hydrogenator was constructed, suitable for use with capillary columns by using the heated injector port of a GC as the catalytic reactor. Ten centimetres of the injector end of the capillary column was straightened, stripped of phase and loaded with approximately 0.5 mg of palladium on charcoal catalyst. Hydrogen carrier gas was used. The system is shown in Figure 20.

6.1.2.1 Verification of the Technique

As a trial 0.1 µℓ aliquots of a 4% solution of safrole in

chloroform was injected at various temperatures and carrier gas flow rates and the reaction products monitored by EI MS.

It was found that by using 100% hydrogen as carrier gas at a flow rate of 3 mls per minute and a catalyst injector temperature of 190° to 210°C almost complete hydrogenation of individual compounds occurred.

On-line hydrogenation of the major components of the oil of Atherosperma moschatum, i.e. safrole and methyl eugenol was also accomplished using the same conditions. The total ion current trace is shown in Figure 21. The mass spectrum of the non-hydrogenated compounds, safrole (scan 706) and methyl eugenol (scan 822) are given in Figure 22 together with the mass spectra of the corresponding hydrogenated compounds, scan 688 and scan 814. Safrole (M^+162) reacted to give dihydrosafrole (M^+164) and methyleugenol (M^+178) reacted to dihydro methyl eugenol (M^+180).

These trials prove the presence of one olefinic double bond in each of the compounds and demonstrated that the technique was applicable to on-line hydrogenation of major components in an essentail oil.

6.1.3 Results and Discussion

Hydrogenation of high boiling compounds present in L. lanigerum

A GC-MS run of the crystalline fraction of L. lanigerum, which contained the two major unidentified compounds, was made. The TIC trace was recorded in Figure 23, and shows isolates No. 2 and 3.

The same fraction was then subjected to on-line hydrogenation GC-MS and the TIC trace of the reaction products was also recorded in Figure 24.

The corresponding spectra are shown in Figure 25 together with their non-hydrogenated spectra.

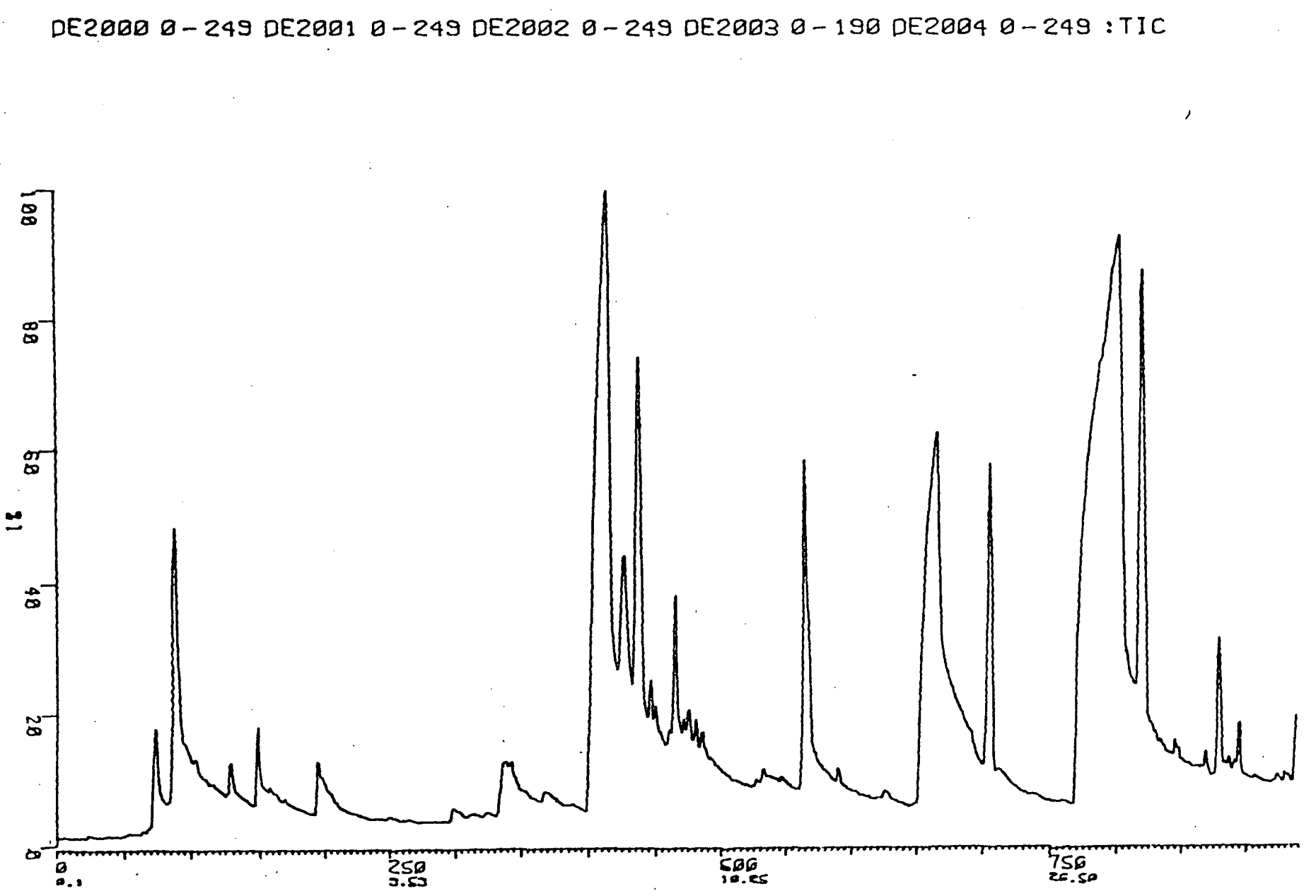


Figure 21. Total ion current trace of pre-column hydrogenator GC-MS of whole oil of A. moschatum CW-20m SCOT column H_2 carrier gas, Temperature programmed 60-180° at 4°C per minute.

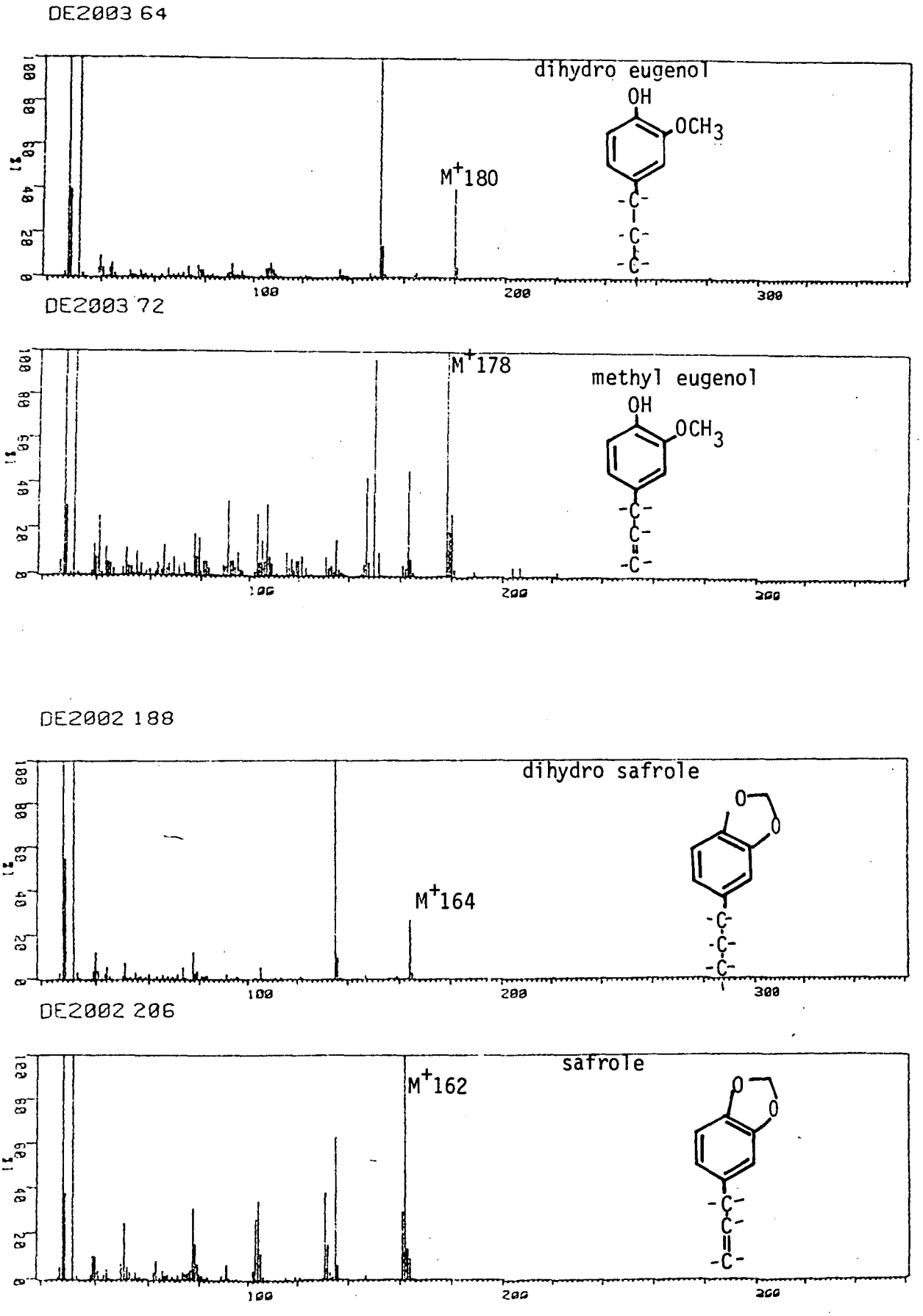


Figure 22. Mass spectra of original and hydrogenated compounds from TIC trace

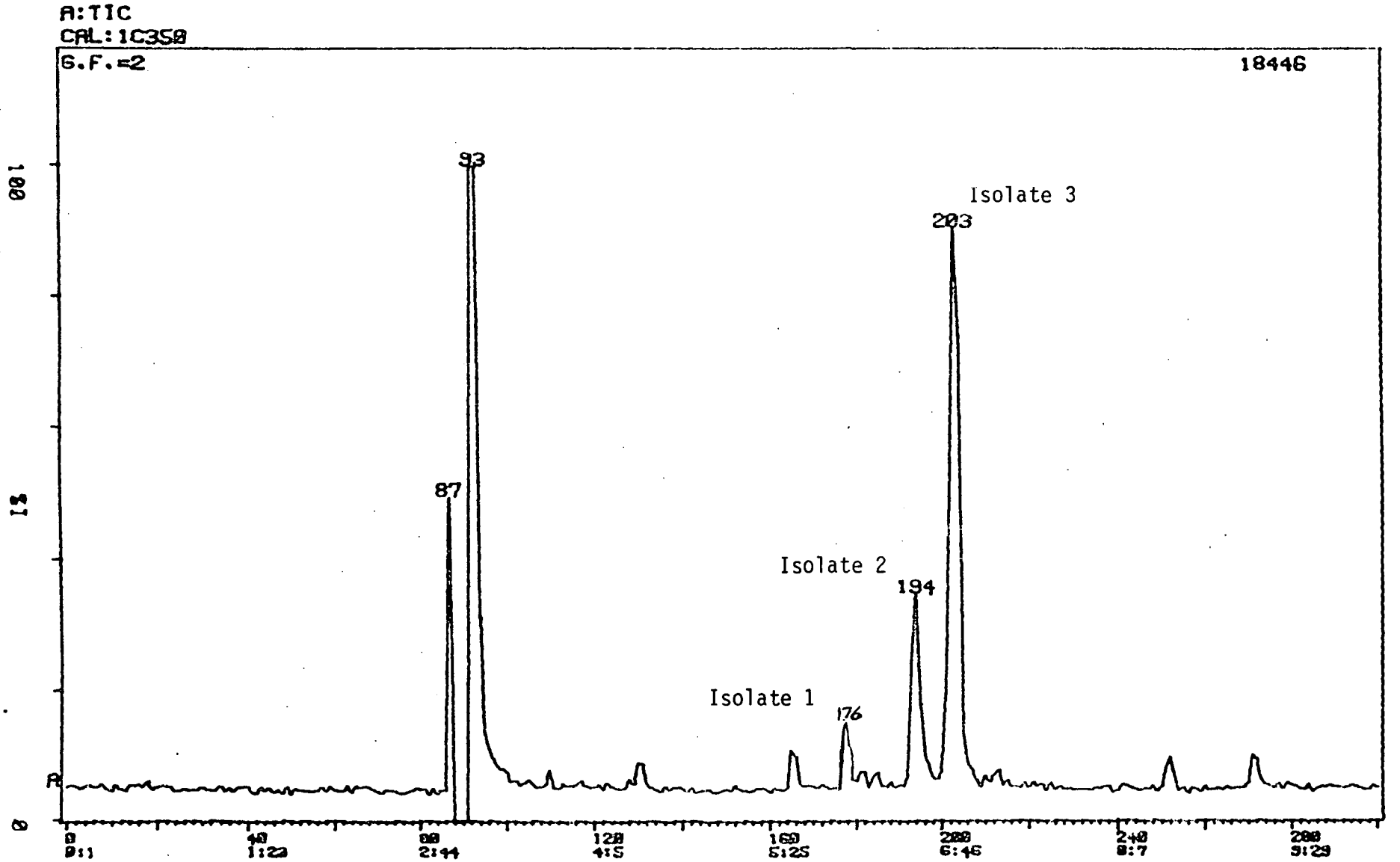


Figure 23. Total ion current trace of crystalline fraction of L. lanigerum OV-1, phase 210°C· isothermal Hydrogen carrier gas.

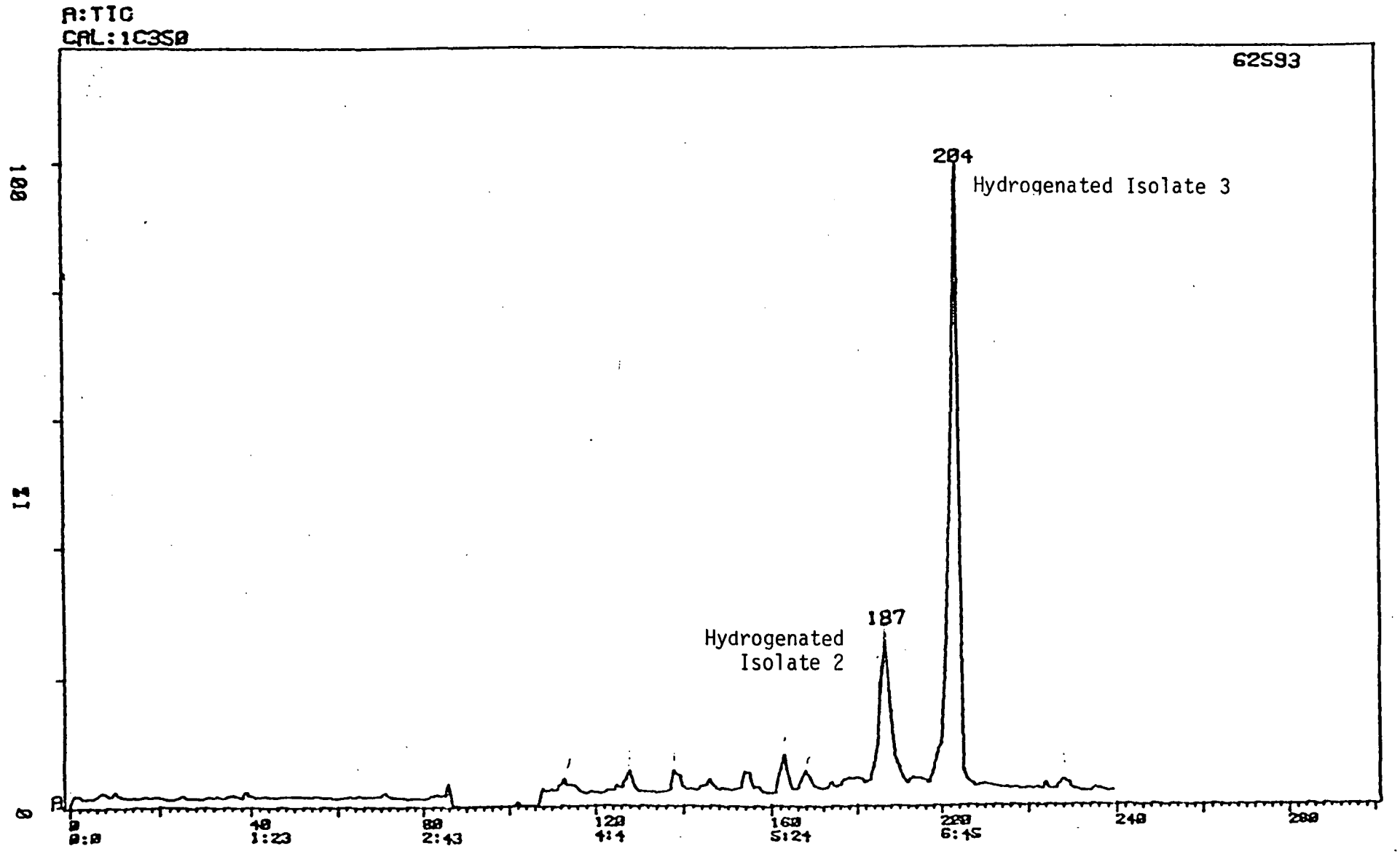


Figure 24. Total ion current trace of the on-line hydrogenated crystalline fraction of L. lanigerum, OV-1 phase, 210°C isothermal, hydrogen carrier gas.

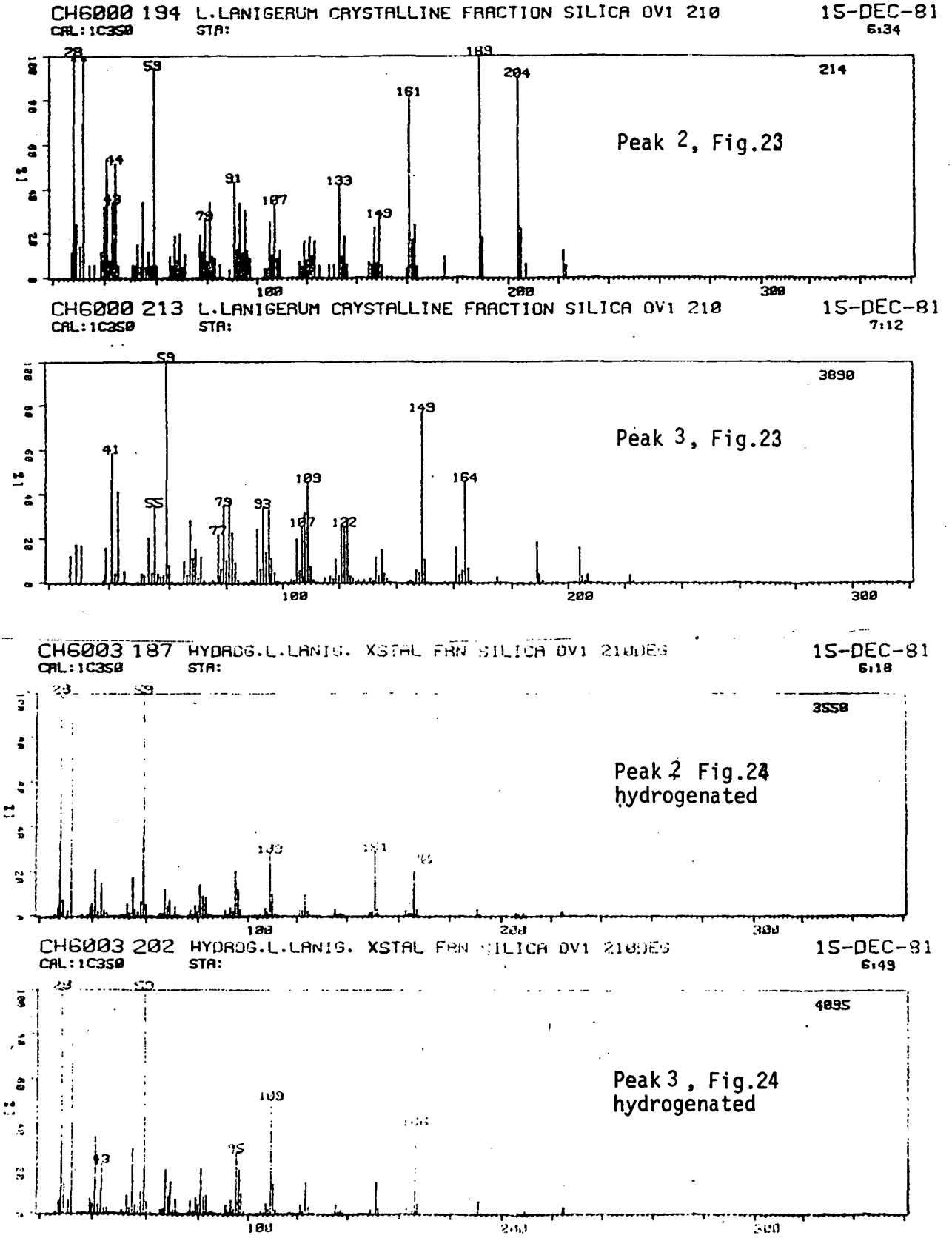


Figure 25. Mass spectra of hydrogenated and non-hydrogenated compounds from Fig.23 and Fig.24

Although the parent ions of all compounds was weak, it could be assumed from the hydrogenated and non-hydrogenated spectra that only one readily hydrogenated double bond was present in both compounds.

A comparison of the areas of the hydrogenated (Figure 24) and non-hydrogenated (Figure 23) peaks showed that the ratios of the reaction products was not consistent with that of the unreacted compounds. This led to the possibility that either compound when hydrogenated may have contributed to either of the hydrogenated compounds.

To verify this, both peaks were collected with the aid of the GC effluent collector as described in Chapter 6, rinsed from the capillary with a few μℓ of ethanol and hydrogenated in the GC-MS system. Isolate No.2, Figure 23, reacted to give two compounds in the ratio 50:50 approximately, whilst Isolate No.3, Figure 23, reacted to give the same two compounds in the ratio of 25:75 approximately.

6.1.4 Conclusions

It was concluded that the two unknown compounds are structural isomers containing one carbon-carbon double bond.

6.2 Carbon Skeleton Chromatography-Mass Spectrometry of Sesquiterpene Compounds in the Oil of L. lanigerum

6.2.1 Introduction

Information on the basic carbon skeleton may assist in the positive identification of an unknown compound.

By using a platinum or palladium catalytic reactor either on-line or off-line, with hydrogen as the carrier gas and temperatures in the vicinity of 250°C, compounds may undergo hydrogenation, dehydrogenation or hydrogenolysis to form the parent hydrocarbon skeleton and/or its next lower homologue (196).

With this technique as little as a few nanograms of a compound

may be reacted and the breakdown products monitored by mass spectrometry.

Because the technique is applicable to a wide variety of compounds, variations in conditions may be necessary to obtain the correct products, i.e. no cracking of carbon-carbon bonds should occur.

6.2.2 Experimental

On-line carbon skeleton chromatography of the two main sesquiterpene compounds in the crystalline fraction of the oil was attempted by injecting 0.1 μℓ amounts into the hydrogenator-injector port of the GC. A catalyst temperature ranging from 250°C to 300°C was used. A neutralized palladium on charcoal catalyst was used in order to prevent cracking of carbon-carbon double bonds.

A complicated mixture of compounds was noted which eluted as a very broad peak. The mass spectrum recorded at various intervals of the total ion current trace showed a predominance of ions similar to the dihydrocompounds found in the reaction products of the hydrogenation experiment.

By variation of the conditions such as carrier gas flow rate, reactor temperature and the amount of catalyst, little improvement was noted in the performance of the reactor system and no assessment of the carbon skeleton of each compound was able to be made.

An off-line reaction technique was also investigated by using samples collected using the GC effluent collector previously described in Chapter VI.

A few milligrams of neutral palladium on charcoal catalyst were introduced into the capillaries so as to deposit onto the collected droplets. These capillaries were then filled with hydrogen, the ends flame sealed and then placed in a tube furnace at temperatures ranging from 250°C to 300°C for one to five minutes.

The reacted sealed capillaries were then placed in a capillary breaker attachment to the GC-MS system. The resulting reaction products eluting on an OV-101 column were monitored by EI MS.

6.2.3 Results and Discussion

The major reaction product resulting from the carbon skeleton chromatography of the two sesquiterpenes was the hydrocarbon 4αH, 5αH-eudesmane (Figure 26).

The chromatography was extremely poor in that incomplete reaction was noted and a mixture of compounds eluted as a broad peak. The major compounds being eudesmane and the dihydro derivatives of the original compounds.

By changing catalytic conditions and reaction times a more complete reaction may have been possible. Variations in proposed conditions have been noted (194, 195, 196), and further experimentation in this area is necessary.

A different injector technique may also aid in better chromatographic separation of the reacted components. Stanley et al (194) proposed the use of an elution-cold trap-rapid heating injection technique whereby all reaction products would be swept onto the column instantaneously.

6.2.4 Conclusions

Although more experimentation in catalytic conditions is required in order to form the correct reaction products, and better methods of sample introduction are needed, the technique was able to show that the basic carbon skeleton of both compounds investigated was the eudesmane skeleton.

6.3 Chemical Subtraction GC for functional group characterization in the high boiling point sesquiterpene compounds of L. lanigerum

6.3.1 Introduction

Reaction loops or pre-columns containing a range of chemicals have

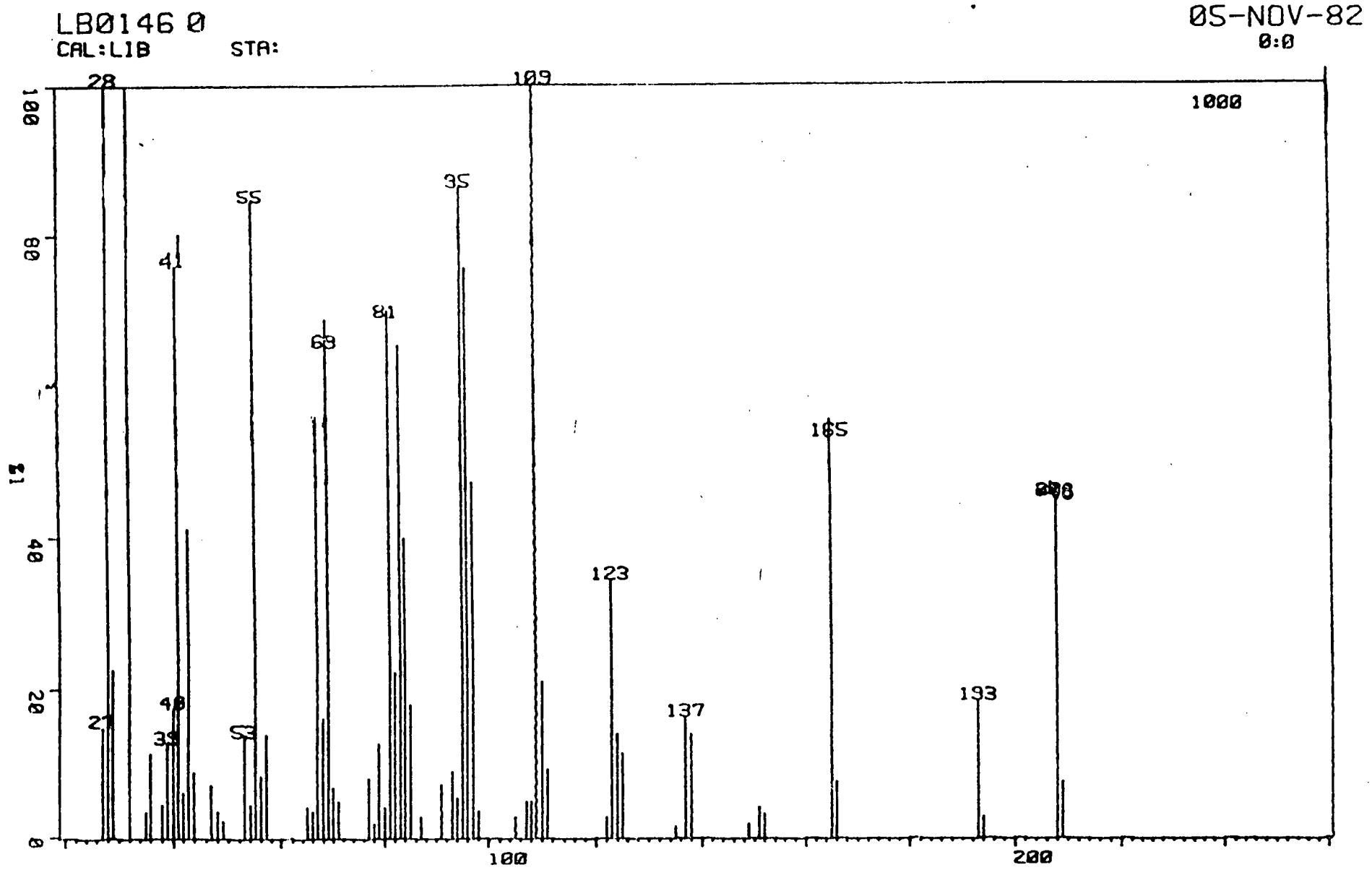


Figure 26. Mass spectrum of carbon skeleton of both compounds present in crystalline fraction of L. lanigerum.

been used for the subtraction of compounds containing a specific functional group. This aid to identification has been used by many workers and is reviewed in Chapter 1.

With packed columns it is possible to detect the functional groups in 0.1 to 1 µg of component in a mixture (122). By using subtraction loops manufactured by coating reagents onto a section of thin walled capillary columns, Kalo (197) has used the technique for ancillary identification of nanogram amounts of alcohols, aldehydes and ketones.

Previous investigation of the sesquiterpenoid compounds in L. lanigerum by Ayling (176) using fractionation on "florisil" led him to the supposition that they were oxygenated. Interpretation of their mass spectra indicated that an alcohol group may be present.

It was thought that an investigation of these compounds by means of subtraction columns could lead to positive identification of their functional groups.

6.3.2 Experimental

The SGE unijector assembly, packed with the various reagents, coupled to a Tracor 550 GC was used for all experiments.

Subtraction reagents were prepared by coating chromosorb W HP 80-100 mesh with saturated solutions of FFAP (free fatty acid phase) in chloroform, 10% benzidine in dichloromethane and 2% boric acid in dimethylformamide. The solvents were removed using a rotary evaporator. 400 milligrams of the coated phase containing approximately 100 ng of the reagent was placed into the glass lined injector assembly of the GC. Before the reagents were used they were conditioned at 200°C for 3 hours in the injector under a flow of nitrogen carrier gas. The GC was used in the split-less mode and the reactor-injector temperature was maintained at temperatures of 150-200°C. For the

trial, 10 ng of a range of model compounds (aldehydes, ketones and alcohols) was injected.

Using these same conditions the unidentified sesquiterpene compounds were also reacted in the reactor-GC-system.

6.3.3 Results and Discussion

The efficiency and selectivity of the compounds was studied by injecting individually the model compounds listed in Table 20. The injector temperature was varied within the ranges listed above, and the GC oven was maintained at 180°C.

The FFAP reactor was found to react with and subtract most aldehydes in mixture I. Whilst all chromatograms showed some residual amounts of aldehydes present, by variation and conditions, including the length of column and column loading, a more complete subtraction may have been accomplished. A temperature of 150°C was found to be optimum for this system. Benzidine packed in the reactor was found to subtract both the aldehydes and ketones present in mixture I at a temperature of 190°C. At this temperature, however, the life of the subtractor was limited to only a few hours.

With boric acid coated Chromosorb at 150°C a variety of reaction products was obtained, perhaps due to dehydration. However, at temperatures of 120°C the primary and secondary alcohols were retained in the injector whereas the tertiary alcohols passed through with a broader peak and longer retention times. This is in agreement with previous workers on subtractor-GC experiments (121, 122) who found that tertiary alcohols do not react to form trialkyl borates.

From these preliminary experiments, it was assumed that although the range of trial compounds selected was small and both GC and subtractor conditions may not have been optimized, the technique could be used to verify the functional groups present in the

Table 20

Results of subtraction experiments with an injector system packed with various reagents.

Mixture I

Carbonyl compounds	FFAP	Benzidine	Boric Acid
1-heptanol	- p	-	+
nononal	- p	-	+
1-decanol	- p	-	+
carvone	+	-	+
anisaldehyde	- p	-	+
cinnamaldehyde	- p	-	+
piperonal	+	-	+
benzophenone	+	-	+

Mixture II

Hydroxy compounds			
cyclohexanol	+	+	-
linalool oxide	+		-
1-octanol	+	+	-
linalool	+	+	+ b
borneol	+	+	-
menthol	+	+	-
α-terpineol	+	+	+ b
geraneol	+	+	-
1-decanol	+	+	-
thymol	+	+	+ b

\- = completely subtracted; - p = partially subtracted

\+ = completely retained; + b = retained with broader peak.

unidentified sesquiterpenes of L. lanigerum.

A 0.2 µℓ injection of a 10% solution in chloroform of the three crystalline fraction containing sesquiterpene compounds was chromatographed through all three subtractor reagents. All compounds were not subtracted by either reagent, however definite peak broadening was noted with the boric acid reagent (Figure 27) suggesting that a tertiary alcohol was present.

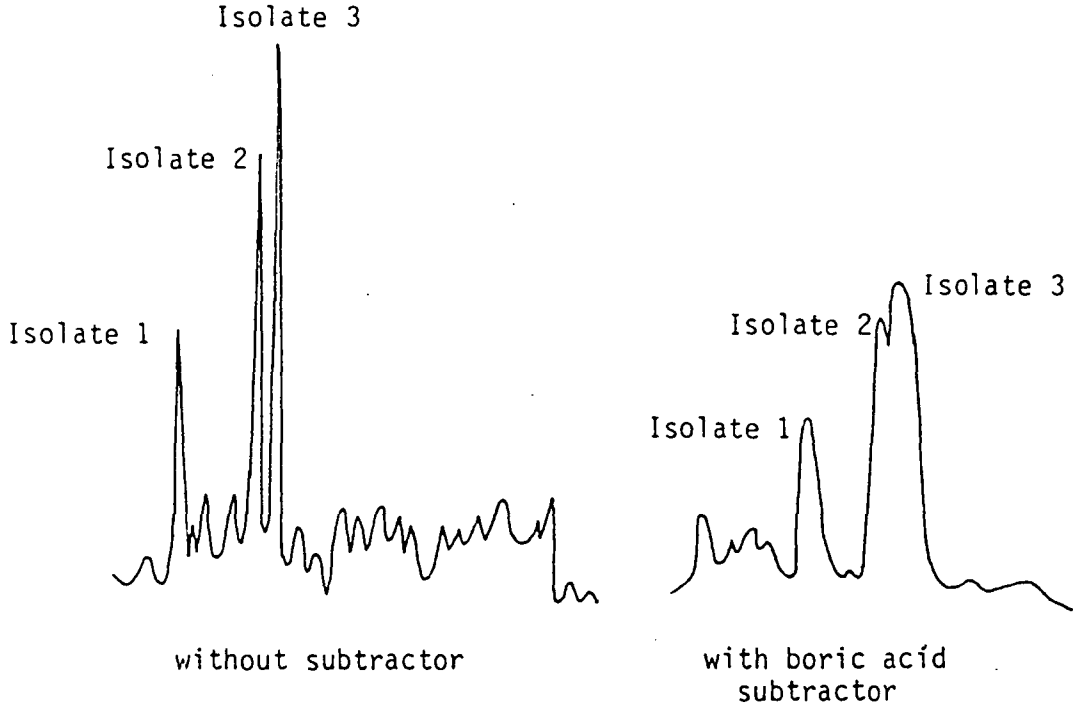


Figure 27. GC Trace of crystalline sesquiterpene fraction from L. lanigerum before and after subtraction with boric acid.

6.3.4 Conclusions

Subtraction-GC has been used to confirm spectral data, that the sesquiterpene has an alcohol group which is tertiary in nature.

CHAPTER 7

7. Infra-red, Raman and NMR Spectroscopic Identification of Sesquiterpene Compounds Isolated from L. lanigerum

7.1 Introduction

To enable identification of the sesquiterpene alcohols present in L. lanigerum, data other than retention indices, reaction GC and mass spectrometry were necessary. The use of the previously described GC fraction collection device would enable NMR, Raman and IR spectra to be recorded on these compounds.

It was also necessary to isolate these compounds by less energetic methods such as TLC and HPLC so as to ensure that the compounds being analysed by GC had not undergone transformations in the separation system. Many reports of artefacts being formed in GC analysis have been noted and reviewed (142).

In this section it is intended to describe the isolation of the pure compounds by these techniques and the comparison of their spectra with those compounds isolated by the more energetic GC capillary cold trap method.

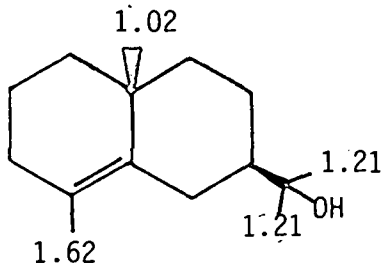
7.2 Experimental

In addition to the two previously isolated fractions of this oil, i.e. Scan 194 (Isolate 2), Scan 203 (Isolate 3), Figure 23, a further peak was collected, Scan 166 (Isolate 1). The MS of this compound showed similarities to the other two isolates and this peak had not been previously identified.

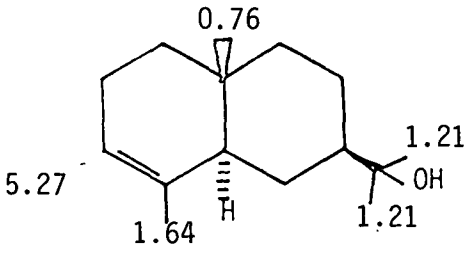
The probe mass spectra of each of the isolates was recorded and it was noted that they were consistent with the spectra of sesquiterpene alcohols.

7.2.1 Raman Spectroscopy

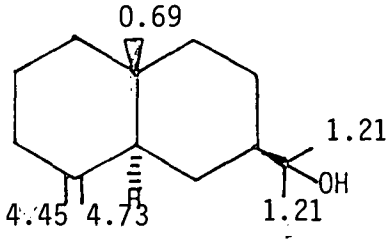
Spectra of each isolate were recorded using exactly the same conditions and methodology as previously described in Chapter 6. These are shown in Figures 28, 29 and 30.



γ-eudesmol



α-eudesmol



β-eudesmol

nmr data for eudesmols

The spectra of a series of authenticated sesquiterpene alcohols were also recorded and it was found that β-eudesmol, Figure 31, gave a spectrum closely resembling that of Isolate 3 (Figure 30). The other two isolates were not able to be matched.

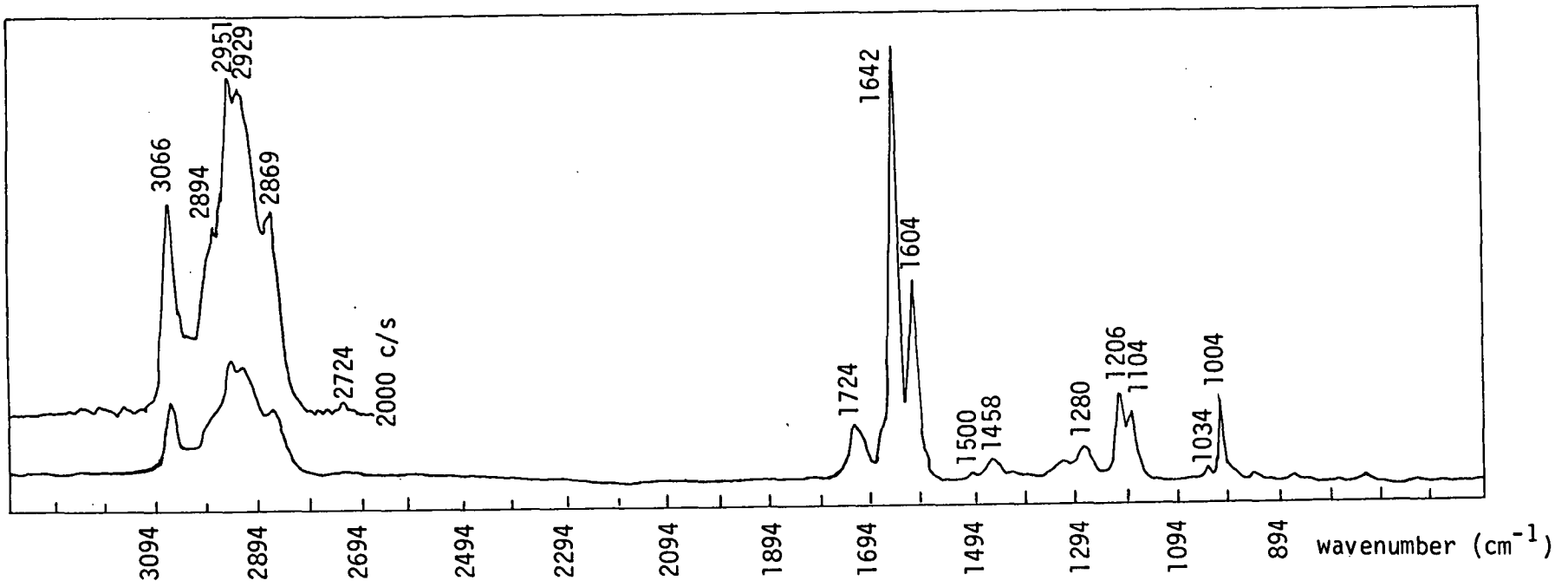


Figure 28. Laser Raman Spectra of Trapped Component No. 1 from Leptospermum laniqerum.

sens. 7000 c/s
SBW 8.0 cm^{-1}
PP 10 secs.

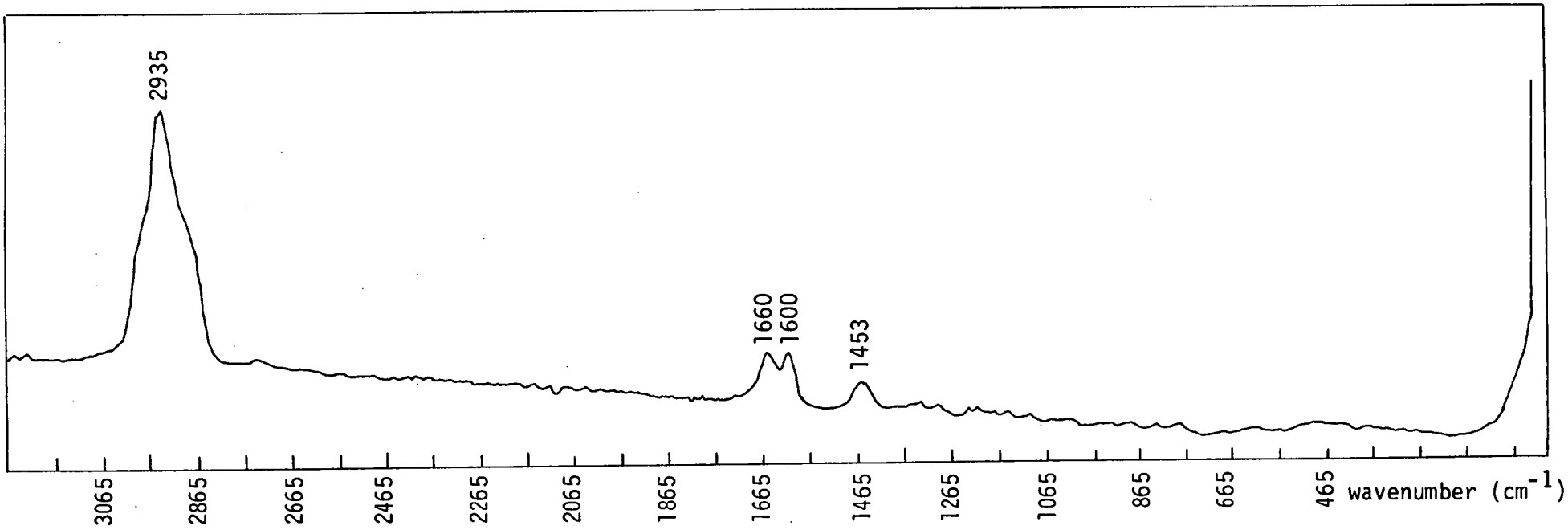


Figure 29. Laser Raman Spectra of Trapped Component No. 2 from Leptospermum lanigerum.

Sens. 1500 c/s
SBW 8.0 cm^{-1}
PP 20 secs.

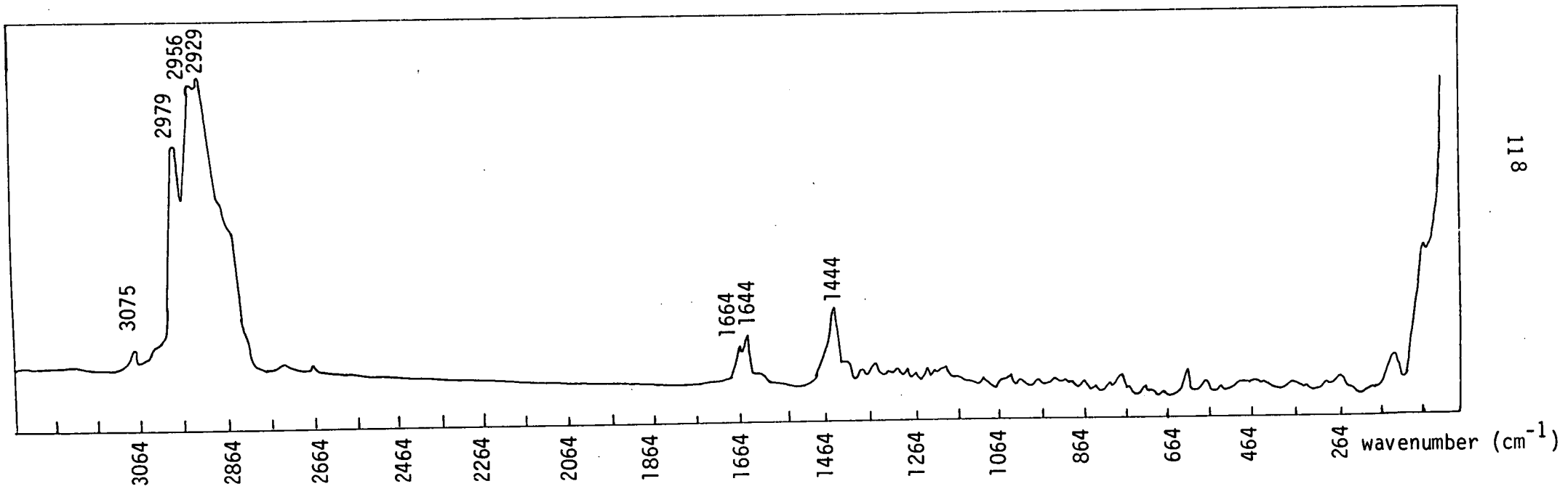


Figure 30. Laser Raman Spectra of Trapped Component No. 3 from Leptospermum lanigerum

Sens. 1000 c/s
SBW 8.0 cm^{-1}
PP 20 secs.

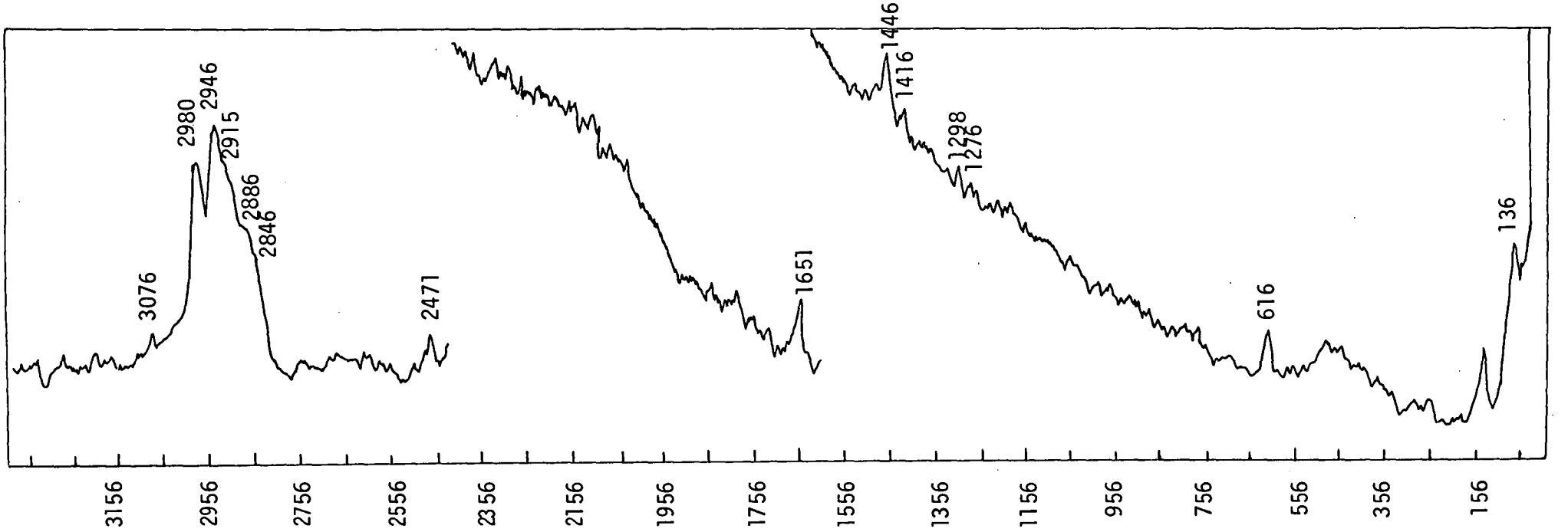


Figure 31. Laser Raman Spectra of an authenticated sample of β-Eudesmol.

Sens. 1600 c/s
SBW 8.0 cm^{-1}
PP 20 secs.

7.2.2 Infra-red Spectroscopy

KBr disc spectra were recorded after elution of the isolates from the capillary tubes with chloroform. A Digilab Fourier Transform FTS-20E spectrometer was used with a resolution of 4 wavenumbers and the spectra are given in Figures 32, 33, 34 and 35.

Although all three spectra were similar, Isolate 3 resembled the spectrum of β-eudesmol (Figure 35) confirming previous laser-Raman data.

7.2.3 NMR Spectroscopy

'Hnmr spectra were recorded on a Bruker HX270 spectrometer from $CDCl_3$ with reference to TMS at 270 mHz using $CDCl_3$ solutions. The spectrum of the three isolates and of a sample of β-eudesmol are shown in Figures 36, 37, 38 and 39. Hara et al. (198) have given NMR data for eudesmols and related compounds and these have been listed previously in this Chapter.

Figure 36, Isolate 1

This isolate gave a spectrum indicating that γ-eudesmol was present, i.e. δ 1.02 (angular methyl) 1.21 (side chain methyl) and 1.62 (methyl attached to double bond). Other shifts were noted that were not associated with this compound. It could be concluded that this isolate was a mixture containing γ-eudesmol. Further GC-MS analysis of this isolate indicated that methyl cinnamate was also present resulting from contamination by an adjacent peak.

Figure 37, Isolate 2

This isolate gave a spectrum indicating possibly a eudesmol type skeleton with key signals at δ 1.02 (angular methyl), 1.62 (methyl attached to double bond), small 1.21 (side chain methyl); some γ-eudesmol may be present.

Figure 38, Isolate 3

This isolate gave the same chemical shifts as did the β-eudesmol

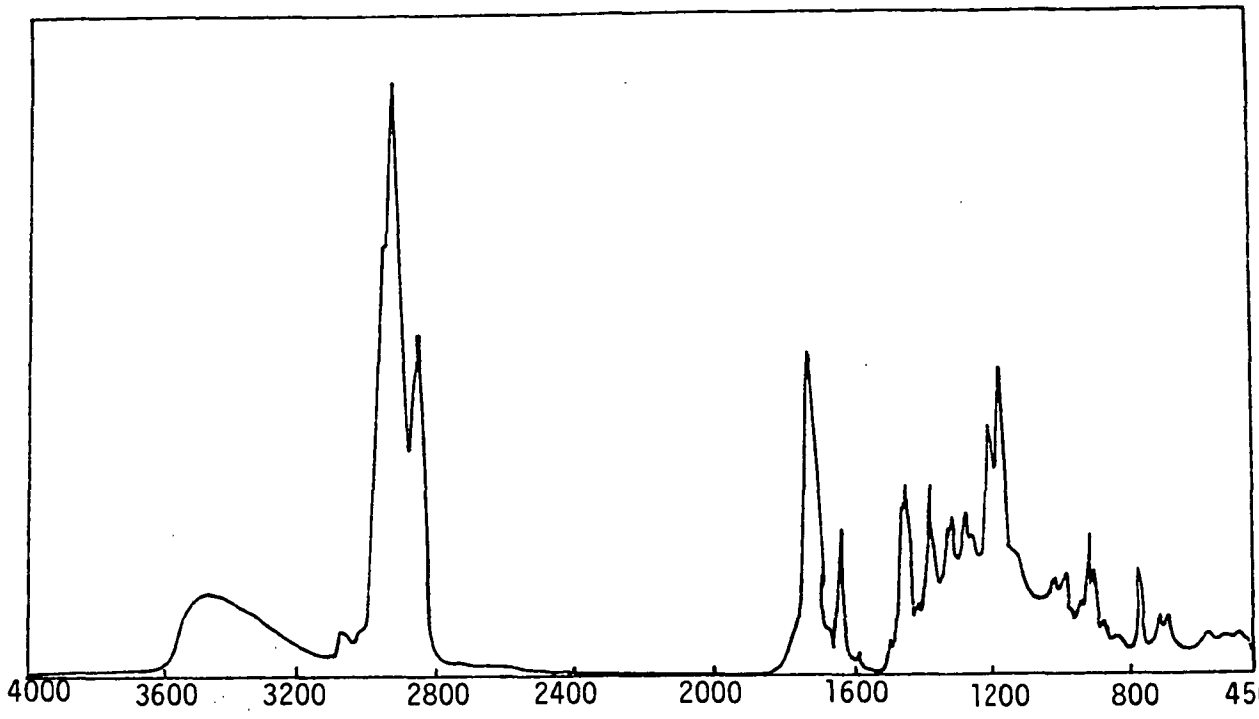


Figure 32.
FTIR spectra of isolate No.1

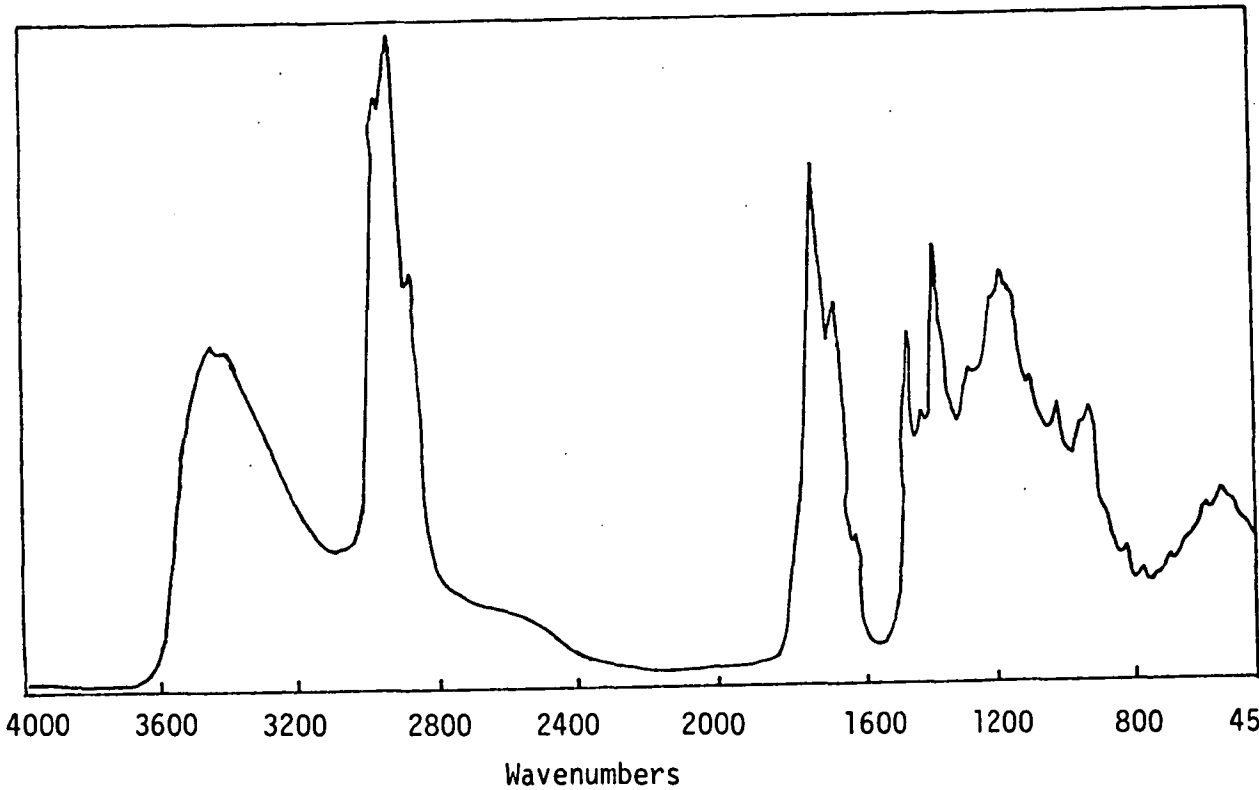


Figure 33.
FTIR spectra of isolate No.2

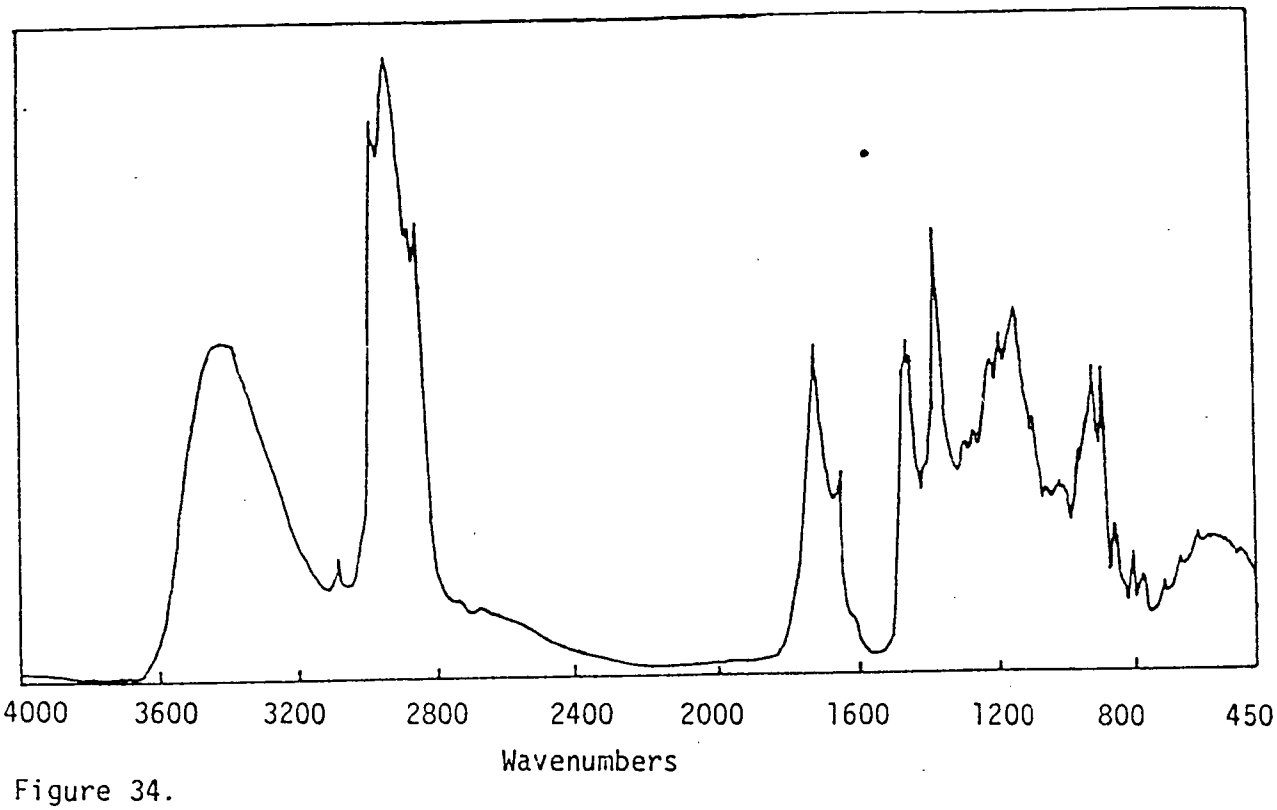


Figure 34.
FTIR spectra of isolate No.3 identified as β-eudesmol

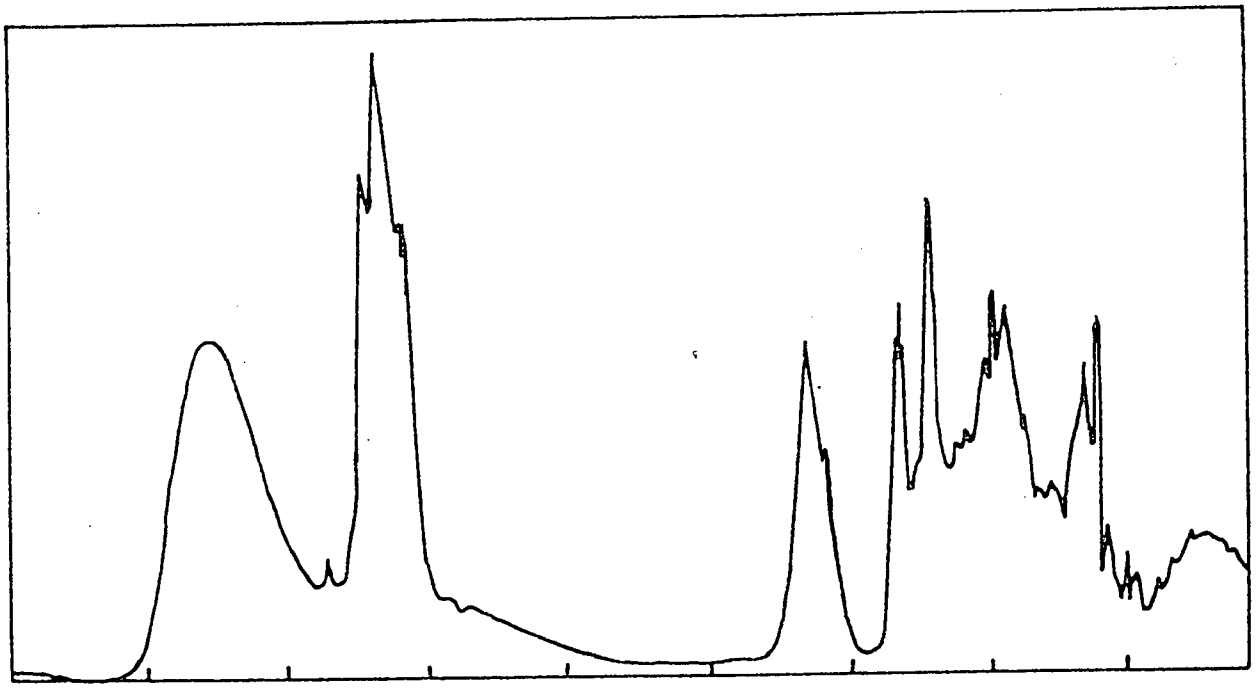


Figure 35. FTIR spectra of authenticated sample of β-eudesmol

authenticated sample, those at δ 0.69 (angular methyl), 1.19 (side chain methyl) and 4.45, 4.73 (vinylic hydrogen) being diagnostic. The resonances at δ 5.3, 0.78 and 1.64 indicated some α-eudesmol impurity, however it may be impossible to separate these isomers with the capillary cold trap technique using a packed column.

Figure 39. Authenticated sample of β-eudesmol (from Dr. R. McQuilkin)

This isolate gave characteristic chemical shifts in agreement with literature data (198), i.e. at δ 0.69 (angular methyl), 1.19 (side chain methyl) and 4.43, 4.73 (vinyl hydrogen). Impurities of α-eudesmol were also found in this sample which was characterized by chemical shifts at δ 0.78, 1.64 and 5.3. This demonstrates the difficulty in obtaining samples of these compounds that are not contaminated with other isomers.

The NMR spectral data of impure isolates collected from packed columns have confirmed that α-, β- and γ-eudesmol are present. However it should be stressed that due to difficulties encountered by sampling GC effluents from a low efficiency packed column it may not be possible to correlate these isolates directly with equivalent peaks on a high resolution capillary column. An alternate separation technique may be necessary in order to obtain pure isolates of these compounds for further NMR investigation.

7.2.4 Isolation and Separation of Sesquiterpene Alcohols by TLC and HPLC

TLC

Preparative TLC to separate sesquiterpene alcohols was attempted by the method of Kirchner (199). Two separate solvent systems were used, 5% ethylacetate/benzene and 5% methanol in benzene on silica gel, Merck type GF254.

The crystalline fraction (220 mg) was dissolved in 1 ml chloroform and stripped onto preparative plates and allowed to develop in the

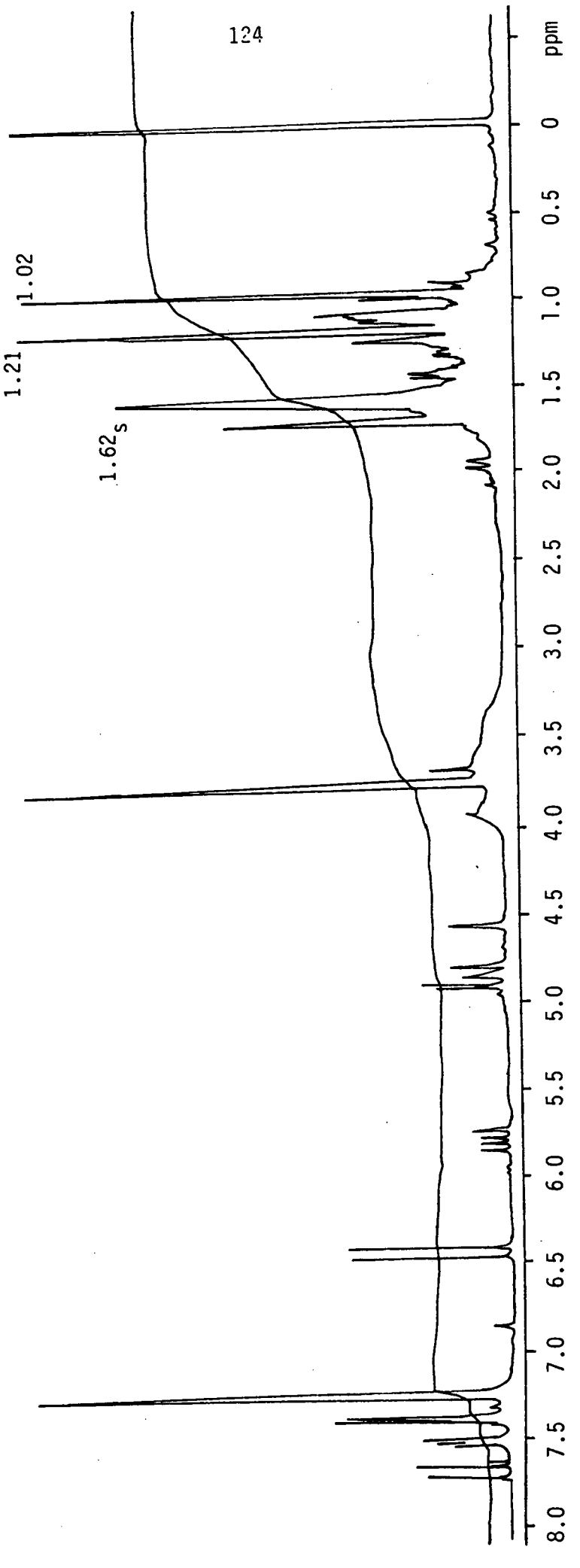


Figure 36. ^{1}Hnmr of Isolate 1 from L. lanigerum and impurities.

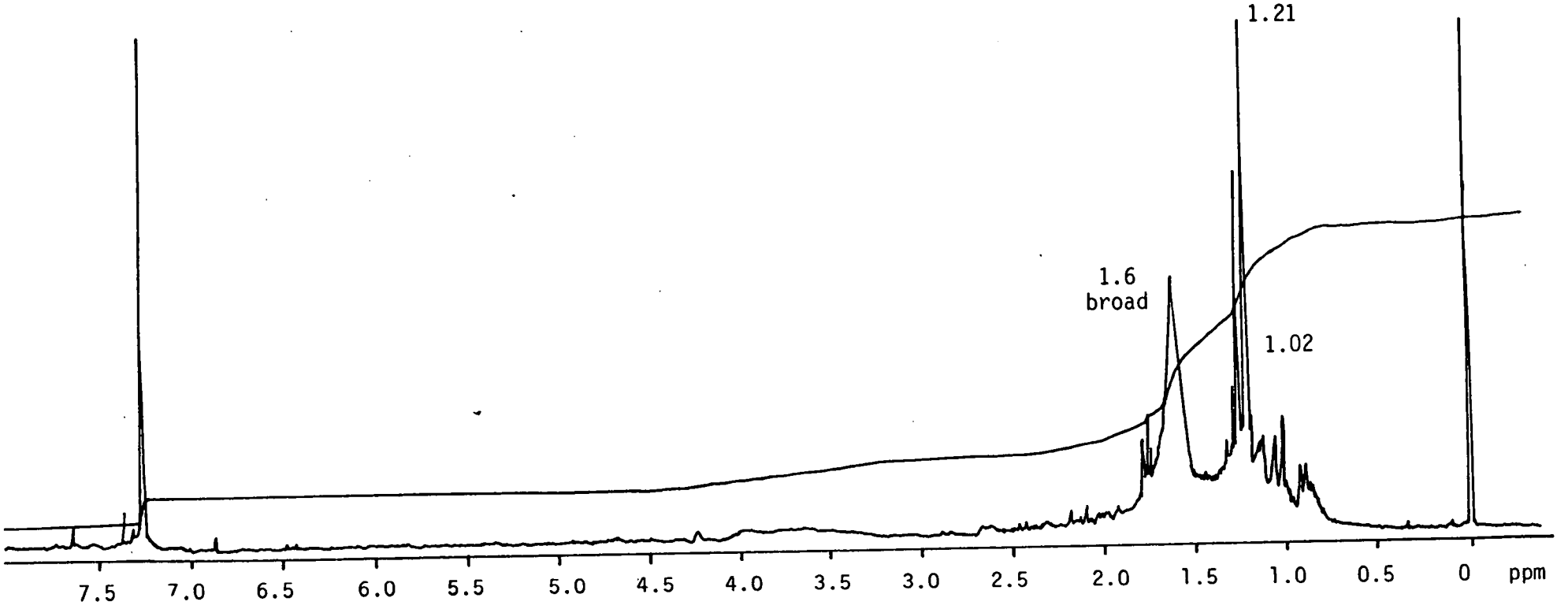


Figure 37. 'Hnmr of Isolate 2 from L. lanigerum

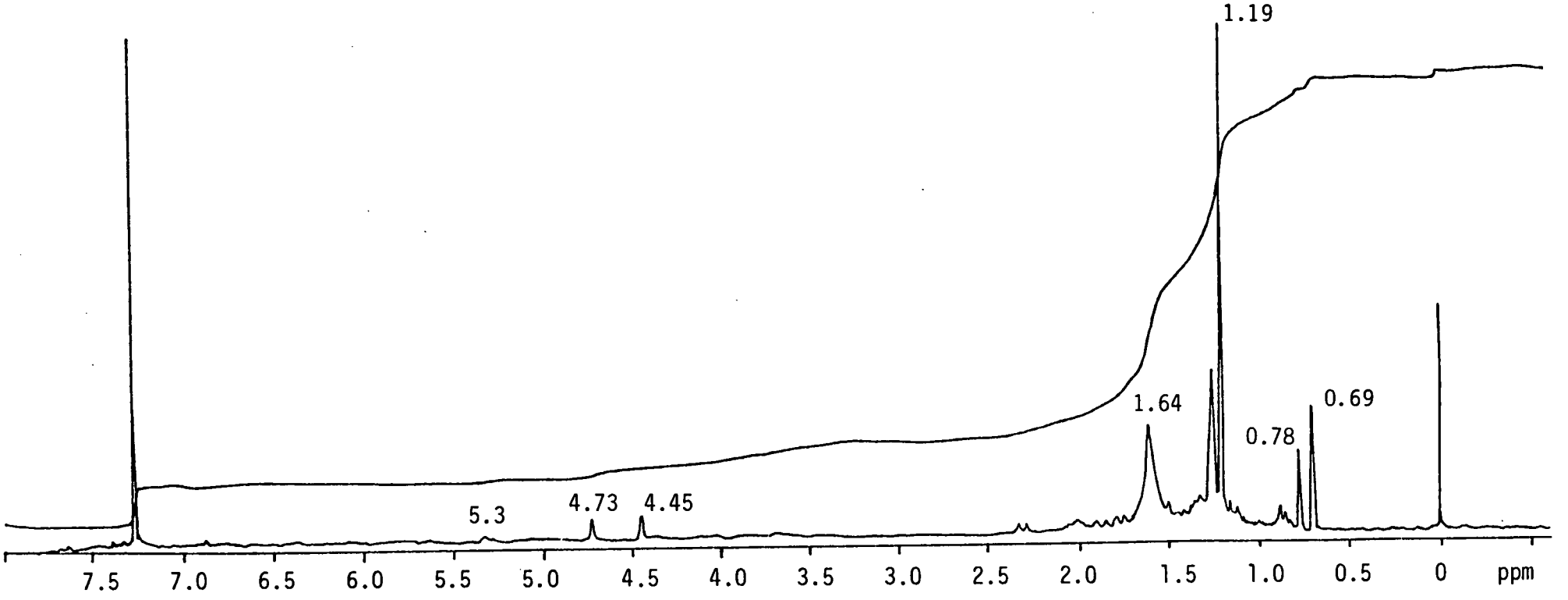


Figure 38. ^1H nmr of Isolate 3 identified as β-eudesmol and α-eudesmol contaminant.

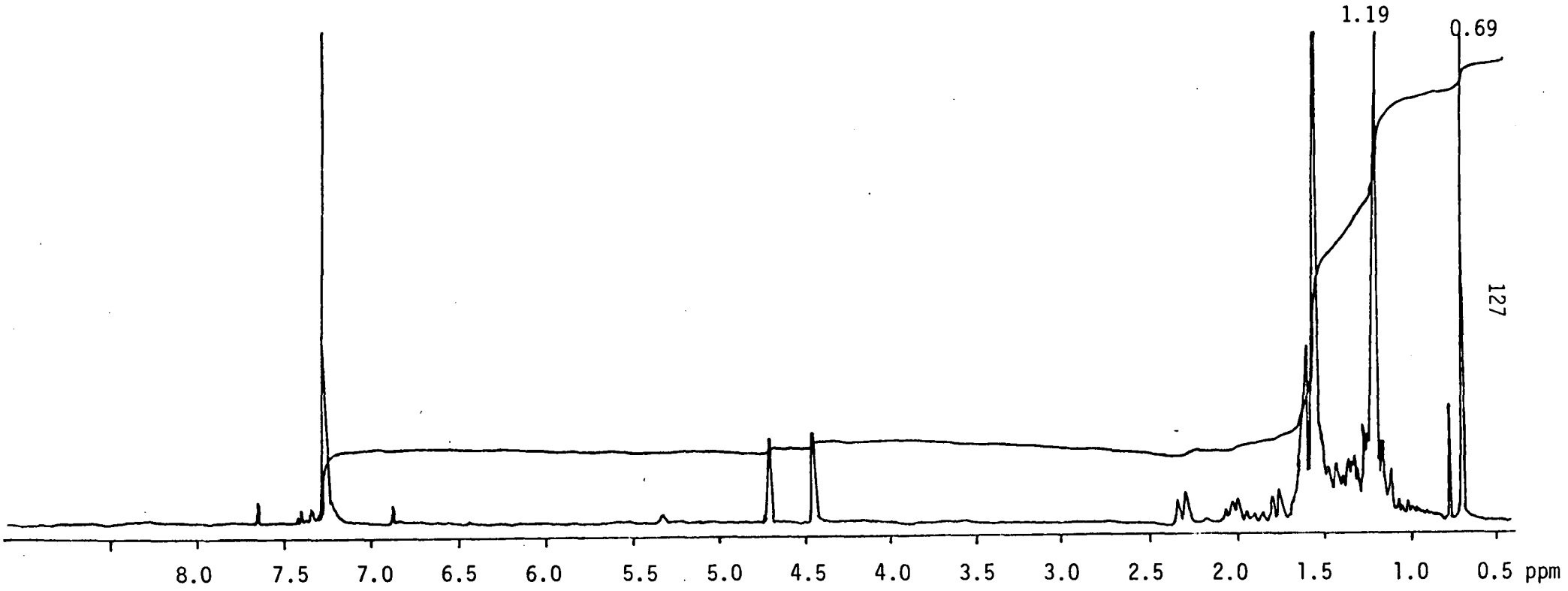


Figure 39. ^1H nmr authenticated sample β-eudesmol , also containing traces of α-eudesmol.

respective solvent systems for about 45 minutes. The bands were identified by UV radiation (254 nm), removed from the plate, extracted with chloroform and evaporated to several microlitres. Although both solvents systems resolved a similar number of compounds, the probe mass spectrum of the major band showed a mixture containing eudesmols. The mass spectra of the other bands indicated lower terpenoids.

Although other TLC solvent systems could have been experimented with, it was considered that HPLC would be a simpler separation technique, due to the ease of changing solvent systems.

HPLC

A Varian Vista HPLC with a 5μ C18 reverse phase column (150 mm x 4 mm) at a pressure of 1500 psi and flow rate of 0.6 mls/minute was used for this experiment.

Varying concentrations of water in methanol were tried as solvent, however only two major peaks could be resolved. With a 50:50 acetonitrile water solvent system it was found that three components were resolved as pre Figure 40.

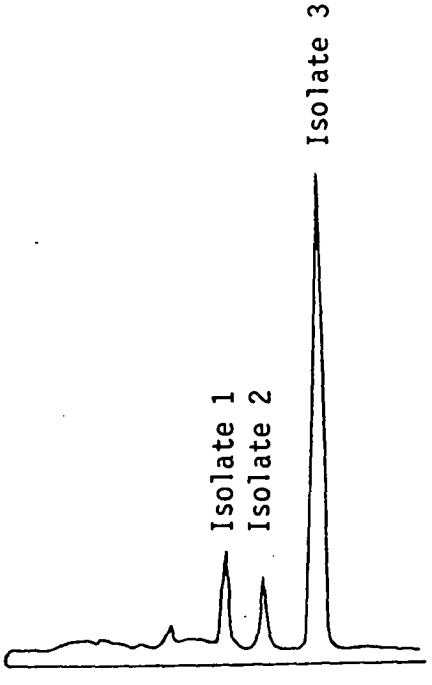


Figure 40. HPLC trace of sesquiterpene alcohols isolated from L. lanigerum

Nanogram amounts of each component were collected as they eluted from the detector and an EI mass spectrum was recorded. These spectra were found to be similar to those previously recorded.

7.3 Conclusions

It could thus be concluded that no alteration of these isolates took place in the GC system. As this separation was carried out on a column of low capacity and high efficiency, insufficient sample was able to be collected for recording NMR spectra. However, a preparative scale HPLC system is being investigated for the isolation of sesquiterpene alcohols.

CHAPTER 8

8. Summary

Many of the compounds present in the essential oils of Leptospermum lanigerum and Atherospermum moschatum were able to be identified by a combination of chromatographic, chemical reaction and spectroscopic techniques. These compounds and the methods by which they were identified are listed in Tables 21 and 22.

Twenty compounds were identified in the oil of A. moschatum, whilst twenty-one compounds were identified in the oil of L. lanigerum. Previous reports on these oils (176) identified 9 compounds in the oil of Atherosperma moschatum and 10 compounds in the oil of Leptospermum lanigerum. All compounds found present by previous workers were confirmed by this investigation.

Retention parameters were found to be of little value for the identification of unknown compounds. It is indicated in Tables 21 and 22 where identification by retention parameters was found to be unreliable and conflicted with MS results. Retention data can, however, be considered satisfactory for screening of components of essential oils prior to examination by other methods.

Gas chromatography coupled with mass spectrometry and subsequent computer analysis was used to identify many of the compounds investigated. It was also used to confirm or reject proposed identifications by retention data.

A system has been described for the rapid collection of nanogram amounts of components eluting from a GC. This enabled further spectroscopic and chemical reaction data to be obtained on

unidentified compounds. Although the technique has limitations with respect to contamination and detection limits, it can be considered an alternate technique where expensive on-line GC spectroscopic instrumentation is not available.

A simple on-column hydrogenator for use with capillary columns was developed and found to be useful for the determination of the number of readily hydrogenated double bonds in a compound.

Gas chromatography - mass spectrometry in conjunction with chemical reaction techniques such as hydrogenation, carbon skeleton chromatography and subtractive reactions have been described that may assist in the structural elucidation of compounds.

These techniques used in conjunction with FTIR, NMR and Raman spectroscopy were found necessary in order to identify compounds not previously identified by mass spectrometry.

Future developments in the analysis of essential oils in this laboratory could include the development of on-line GC-FTIR techniques whereby isomeric compounds, which are sometimes difficult to identify by MS alone, could be identified. Semi-preparative scale HPLC is also being investigated as a technique for resolving difficult to separate isomers such as the eudesmols so that NMR data can be determined.

Table 21 Identification of components in the essential oil of *A. moschatum* by various techniques.

Compound	RRT	Kovats Indices	Mass Spectrometry	Raman Spectrometry
α-pinene	⊕	+	+	+
camphene	⊕	+	+	+
β-pinene	⊕	+	+	+
myrcene	⊕	+	+	+
(-)-limonene	+	+	+	+
1,8-cineole	+	+	+	+
p-cymene	+	+	+	+
α-phellandrene		+	+	
trans-ocimene			+	
β-ocimene			+	
fenchone			+	
camphor	+	+	+	+
linallol	+	+	+	+
terpinen-4-ol			+	
borneol	+		+	+
anethole/estragole			+	
safrole	+	+	+	+
bornyl acetate	+	+	+	
eugenol			+	
methyl eugenol	⊕	+	+	+

⊕ indicates where RRT data unreliable and conflicts with MS results.

Table 22 Identification of components in the essential oil of *L. lanigerum* by various techniques.

Compound	Kovats Indices	Mass Spectrometry	Infra-Red	Raman	NMR
α-pinene	⊕	+			
β-pinene	⊕	+			
1,8-cineole	⊕	+			
2-methyl isopropyl benzene		+			
hexenol		+			
linallol		+			
terpinen-4-ol	+	+			
myrtenol	+	+			
citronellyl acetate		+			
α-terpineol	+	+			
methyl cinnamate		+			
methyl cinnamate isomer		+			
2-decanone		+			
geranyl acetate	+	+			
citronellol	+	+			
myrtenol		+			
2-phenylethyl acetate		+			
linalyl acetate		+			
γ-eudesmol					+
α-eudesmol					+
β-eudesmol					+

⊕ indicates where RRT data unreliable and conflicts with MS results.

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APPENDIX 1

Computer program for calculation of Kovats Indices by cubic spline functions.

```
- -

const   ma  : ...=50;
        ma  table=51;
type    t=r'e array [0..ma  ...=] of real;
        f'...ame=array [1...'] of char;
        line=array [1..'0] : char;
var     ...b.c.d.h...w: ...  :
        ...,j...:integer;
        f:...;
        in...out:file of char;
        t:string[line;
        inname.default:f:'...me;

procedure initialise;
var     i,j:integer;
begin   /* initialise */
        for i:=0 to maxtable do
        begin   x[i]:=0; y[i]:=0;
                b[i]:=0; c[i]:=0; d[i]:=0;
                h[i]:=0; v[i]:=0; w[i]:=0
        end
end;    /* initialise */

procedure getname(var name:filename; default:filename; len:integer);
var     instring:line;
begin   /* getname */
        write('Enter filename (',default,'):');
        read(instring);
        for i:=1 to 10 do name[i]:=instring[i];
        if name[1]=' ' then name:=default
end;    /* getname */

procedure printparams;
var     i,j:integer;
begin   /* printparams */
        writeln; writeln('  x      y        b       c       d');
        for i:=0 to n do
        begin   writeln(x[i]:3:3,'      ',y[i]:4:1,'    '
                                          ,b[i]:3:3,
                '       ',c[i]:3:3,'    ',d[i]:3:3)
/*                      ,h[i]:3:3,'     ',v[i]:2:3,'    ',w[i]:3:2,'    ') */
        end;
        writeln; writeln;
end;    /* printparams */

procedure readcalfile;
var     i,j,len:integer;
begin   /* readcalfile */
        len:=-1;
        writeln; writeln('Read calibration file');
        while len=-1 do
        begin   getname(inname,'GCCAL.DAT ',len);
                reset(in,inname,'DAT',len);
                if len=-1 then writeln('File ',inname,' not found. Try again.');
        end;
        i:=0;
        while not eof(in) do
        begin   i:=i+1;
                readln(in,x[i],y[i]);
                writeln(i:3,x[i]:3:3,y[i]:4:1);
        end;
        n:=i;
```

```
        close(inn)
end;    /* readcalfile */

procedure splinefit;
var     i,j:integer;
begin   /* splinefit */
        for i:=1 to n-1 do h[i]:=x[i+1]-x[i];
        h[0]:=h[1]; h[n]:=h[n-1];
        for i:=2 to n-1 do v[i]:=2*(h[i]+h[i-1]);
        v[1]:=2*h[1]; v[n]:=2*h[n-1];
        for i:=2 to n-1 do
             w[i]:=3*( h[i-1]/h[i]*(y[i+1]-y[i]) +h[i]/h[i-1]*(y[i]-y[i-1]));
          w[1]:=3*(y[2]-y[1]);  w[n]:=3*(y[n]-y[n-1]);
        for i:=2 to n do
        begin   v[i]:=v[i]-h[i]*h[i-1]/v[i-1];
                w[i]:=w[i]-h[i]*w[i-1]/v[i-1]
        end;
        b[n]:=w[n]/v[n];
        for i:=n-1 downto 1 do b[i]:=(w[i]-h[i-1]*b[i+1])/v[i];
        for i:=2 to n-1 do
                c[i]:=3*(y[i+1]-y[i])/h[i]/h[i]-(2*b[i]+b[i+1])/h[i];
          c[1]:=0;  c[n]:=0;
        for i:=1 to n-1 do d[i]:=(c[i+1]-c[i])/3/h[i];
          d[n]:=0;
end;    /* splinefit */

procedure results;
var     i,j:integer;
        yy,s,xx:real;
label   10,99;
begin   /* results */
        writeln;
10:     write('Enter x-value: ');
        readln(xx);
        if xx=0 then goto 99;
        if (xx<x[1]) then i:=1 else
          if (xx>=x[n]) then i:=n else
            for i:=1 to n-1 do if xx<x[i+1] then exit;
        s:=xx-x[i];
        yy:=y[i] + b[i]*s + c[i]*s*s + d[i]*s*s*s;
        writeln('                              y=',yy:4:5);
        goto 10;
99:     i:=1
end;    /* results */

begin   /*main */
        k:=0;
        n:= maxvalue;
        initialise;
        readcalfile;
        splinefit;
/*      printparams;     */
        results;
end.    /* main */
```

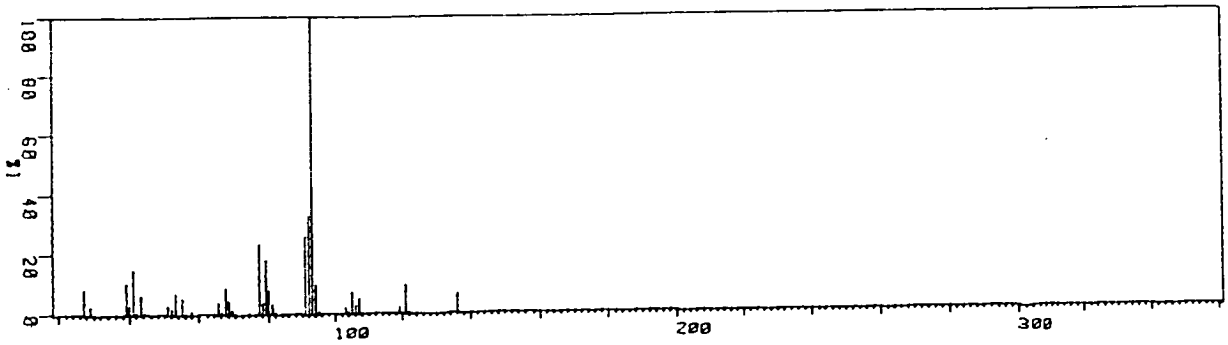
```
program gc;

const   maxvalue=50;
        maxtable=51;
type    table=array [0..maxtable] of real;
        filename=array [1..12] of char;
        line=array [1..134] of char;
var     x,y,b,c,d,h,v,w:table;
        n,i,j,l:integer;
        k:real;
        inn,out:file of char;
        instring:line;
        innname,default:filename;

procedure initialise;
var     i,j:integer;
begin   /* initialise */
        for i:=0 to maxtable do
        begin   x[i]:=0; y[i]:=0;
                b[i]:=0; c[i]:=0; d[i]:=0;
                h[i]:=0; v[i]:=0; w[i]:=0
        end
end;    /* initialise */

procedure getname(var name:filename; default:filename; len:integer);
var     instring:line;
begin   /* getname */
        write('Enter filename (',default,'):');
        read(instring);
        for i:=1 to 10 do name[i]:=instring[i];
        if name[1]=' ' then name:=default
end;    /* getname */

procedure printparams;
var     i,j:integer;
begin   /* printparams */
        writeln; writeln(' x      y        b       c       d');
        for i:=0 to n do
        begin   writeln(x[i]:3:3,'      ',y[i]:4:1,'    '
                                        ,b[i]:3:3,
                '      ',c[i]:3:3,'    ',d[i]:3:3)
/*                      ,h[i]:2:3,'     ',v[i]:2:3,'    ',w[i]:3:2,'    ') */
        end;
        writeln; writeln;
end;    /* printparams */

procedure readcalfile;
var     i,j,len:integer;
begin   /* readcalfile */
        len:=-1;
        writeln; writeln('Read calibration file');
        while len=-1 do
        begin   getname(innname,'GLCAL.DAT',len);
                reset(inn,innname,'DAT',len);
                if len=-1 then writeln('File ',innname,' not found. Try again.');
        end;
        i:=0;
        while not eof(inn) do
        begin   i:=i+1;
                readln(inn,x[i],y[i]);
                writeln(i:3,x[i]:3:3,y[i]:4:1);
        end;
        n:=i;
```

```
        close(i,n);
end;    /* readcalfile */

procedure splinefit;
var     i,j:integer;
begin   /* splinefit */
        for i:=1 to n-1 do h[i]:=x[i+1]-x[i];
        h[0]:=h[1]; h[n]:=h[n-1];
        for i:=2 to n-1 do v[i]:=2*(h[i]+h[i-1]);
        v[1]:=1.5*h[1]; v[n]:=2*h[n-1];
        for i:=2 to n-1 do
             w[i]:=3*( h[i-1]/h[i]*(y[i+1]-y[i]) +h[i]/h[i-1]*(y[i]-y[i-1]));
          w[1]:=3*(y[2]-y[1]);  w[n]:=3*(y[n]-y[n-1]);
        for i:=2 to n do
        begin   v[i]:=v[i]-h[i]*h[i-2]/v[i-1];
                w[i]:=w[i]-h[i]*w[i-1]/v[i-1]
        end;
        b[n]:=w[n]/v[n];
        for i:=n-1 downto 1 do b[i]:=(w[i]-h[i-1]*b[i+1])/v[i];
        for i:=2 to n-1 do
                c[i]:=3*(y[i+1]-y[i])/h[i]/h[i]-(2*b[i]+b[i+1])/h[i];
          c[1]:=0;  c[n]:=0;
        for i:=1 to n-1 do d[i]:=(c[i+1]-c[i])/3/h[i];
          d[n]:=0;
end;    /* splinefit */

procedure results;
var     i,j:integer;
        yy,s,xx:real;
label   10,99;
begin   /* results */
        writeln;
10:     write('Enter x-value: ');
        readln(xx);
        if xx=0 then goto 99;
        if (xx<x[1])then i:=1 else
         if (xx>=x[n]) then i:=n else
          for i:=1 to n-1 do if xx<x[i+1] then exit;
        s:=xx-x[i];
        yy:=y[i] + b[i]*s + c[i]*s*s + d[i]*s*s*s;
        writeln('                          y=',yy:4:5);
        goto 10;
99:     i:=1
end;    /* results */

begin   /*main */
        k:=0.0;
        n:=maxvalue;
        initialise;
        readcalfile;
        splinefit;
/*      printparams;    */
        results;
end.    /* main */
```

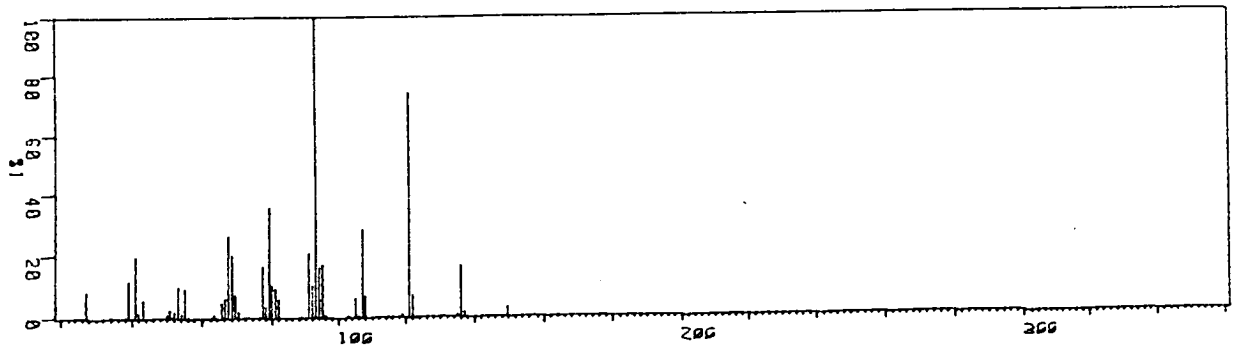
APPENDIX 2

Mass spectra of components of A. moschatum, separated on Carbowax 20m phase with reference to total ion current trace Fig. 9.

DE2200 97



DE2200 114



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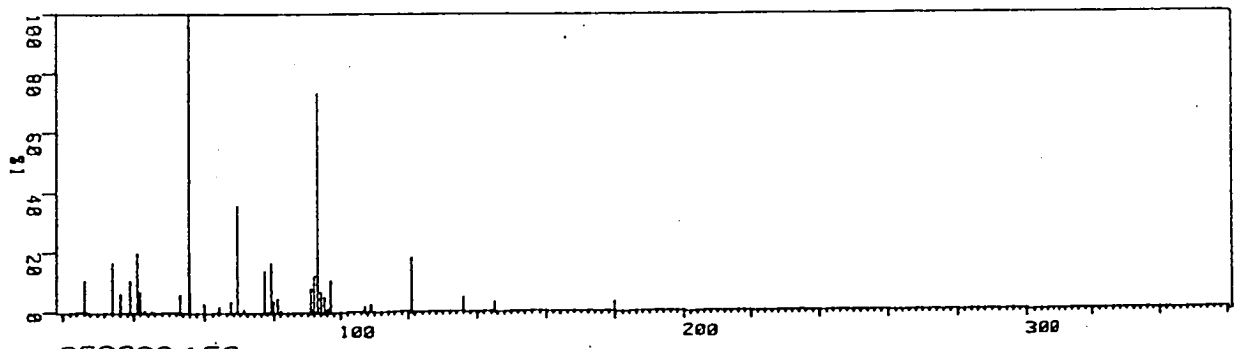

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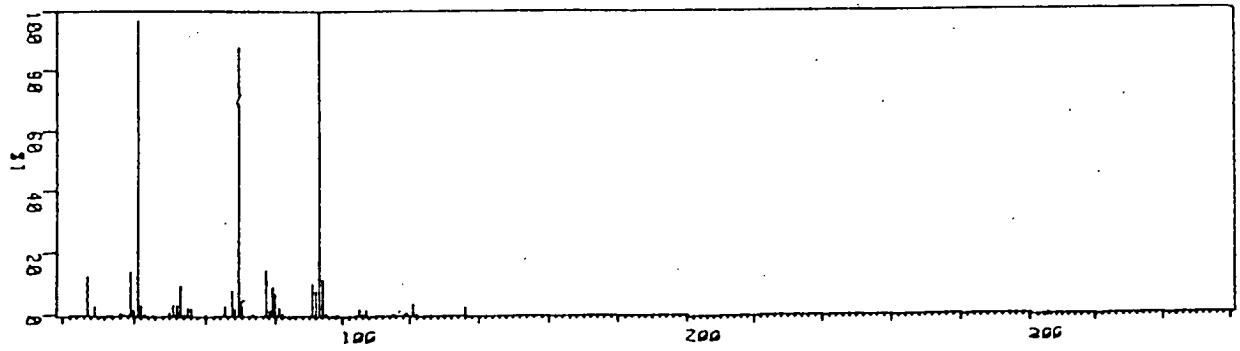

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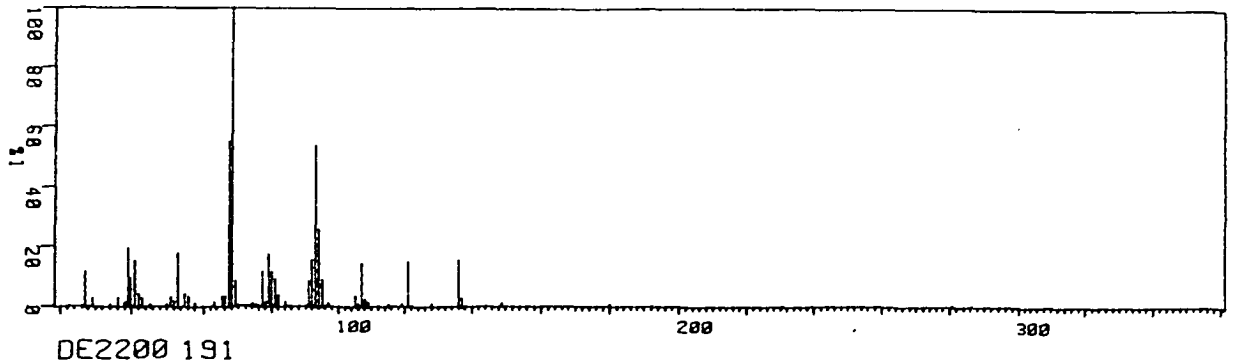

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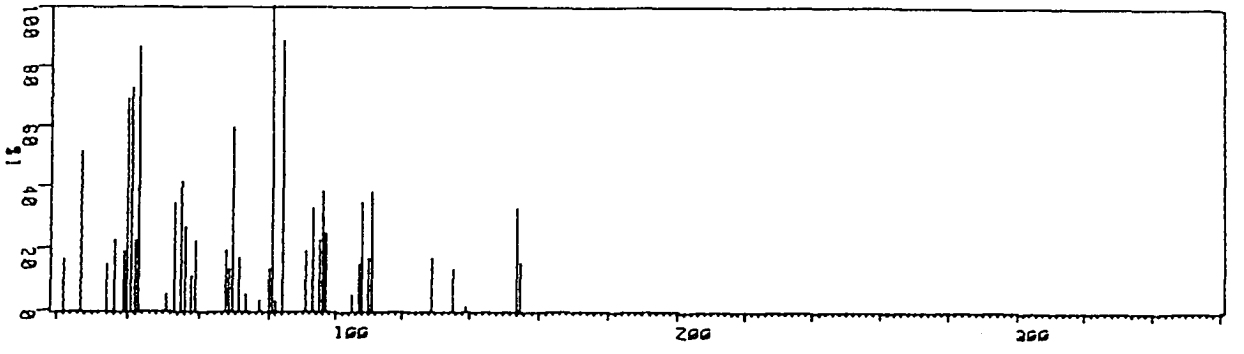

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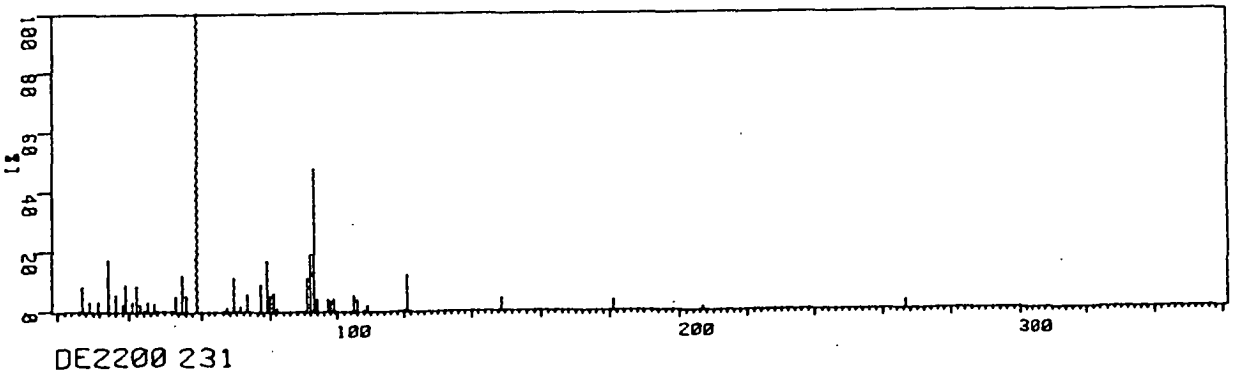

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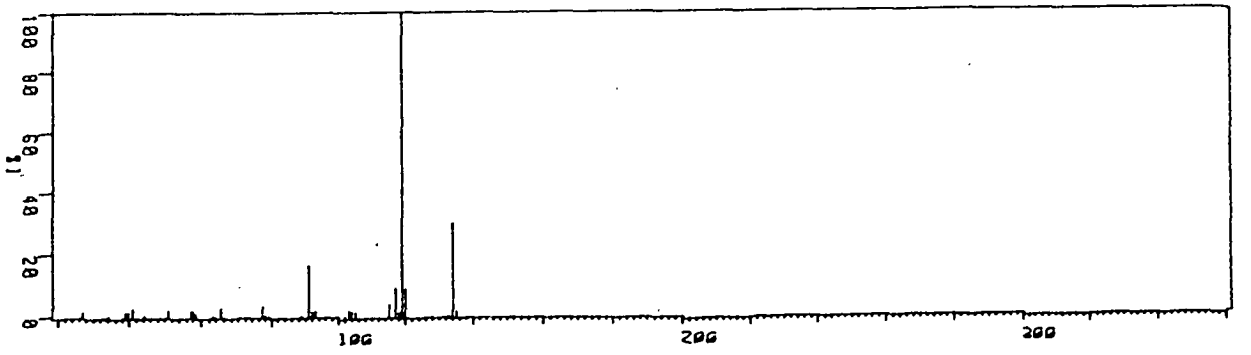

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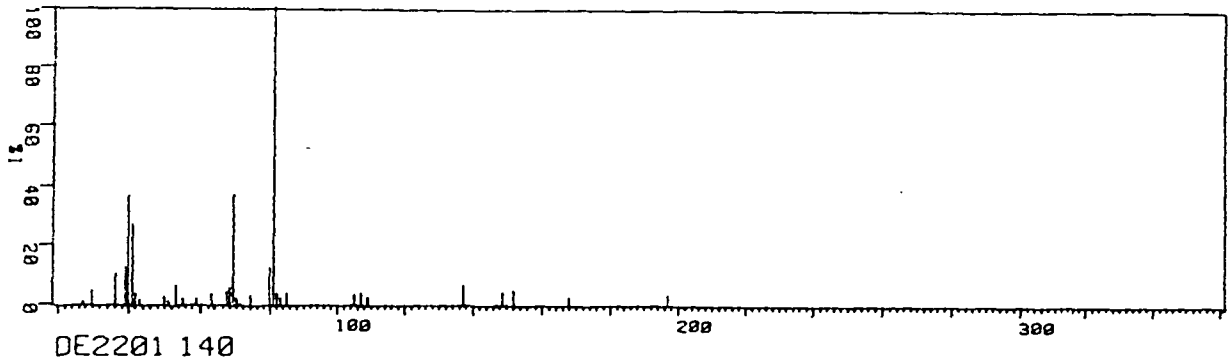

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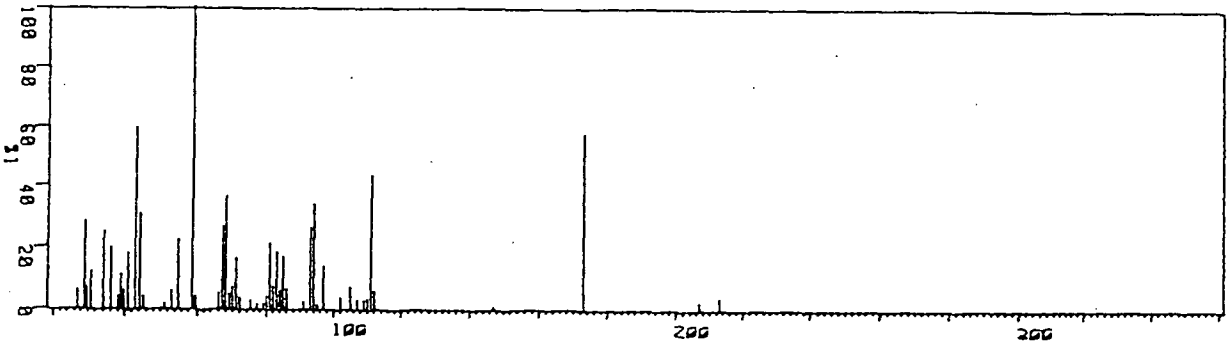

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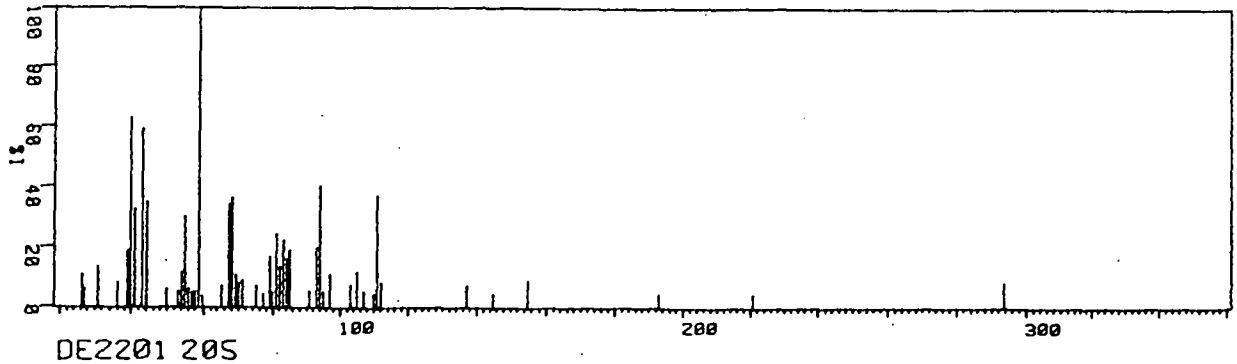

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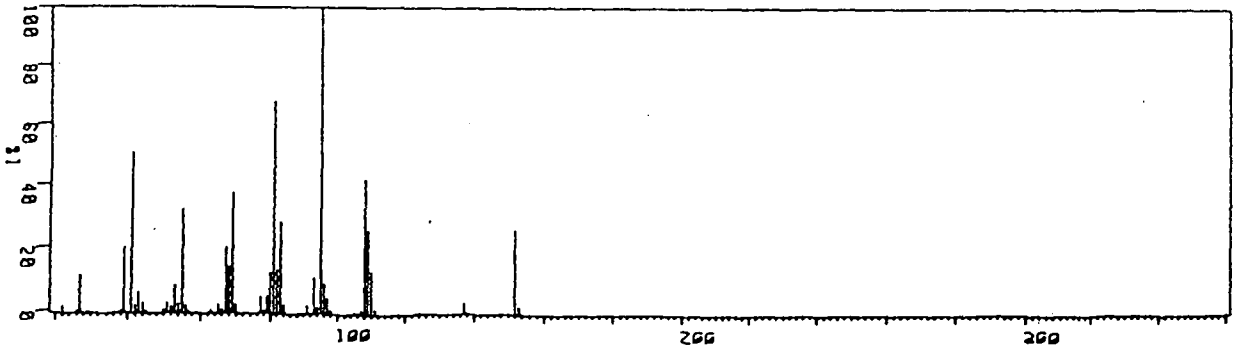

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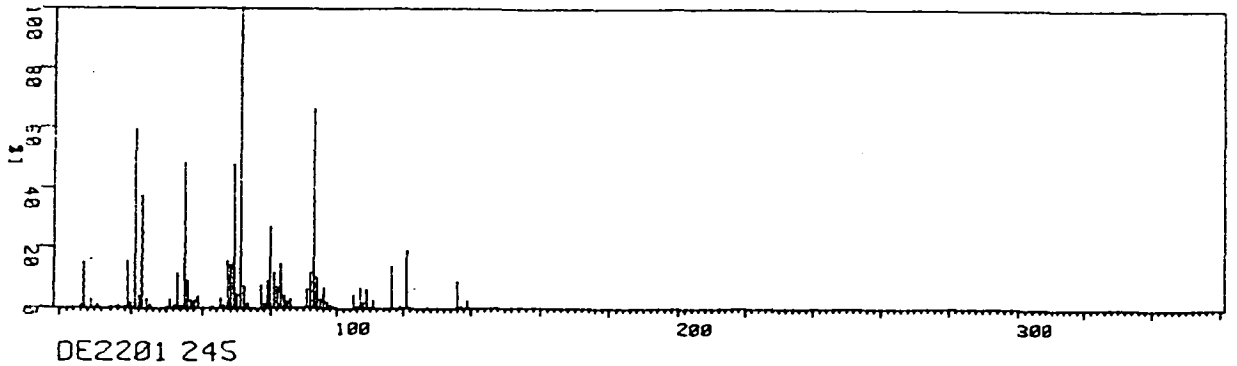

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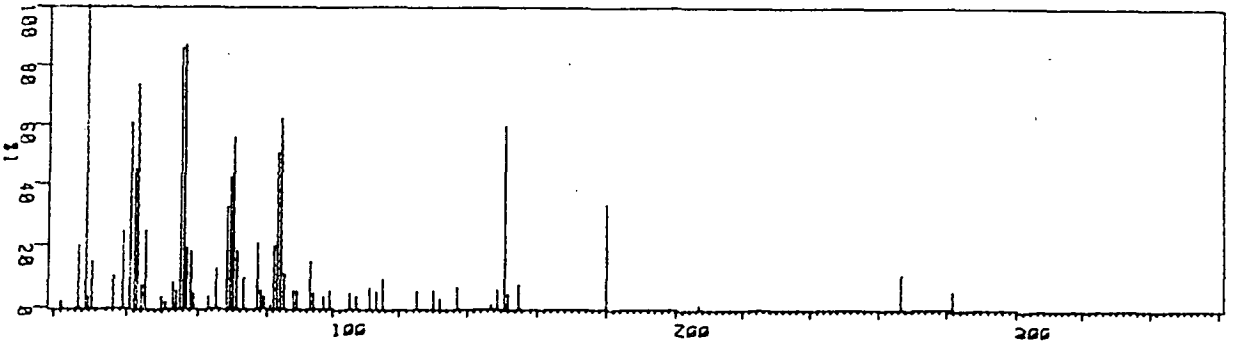

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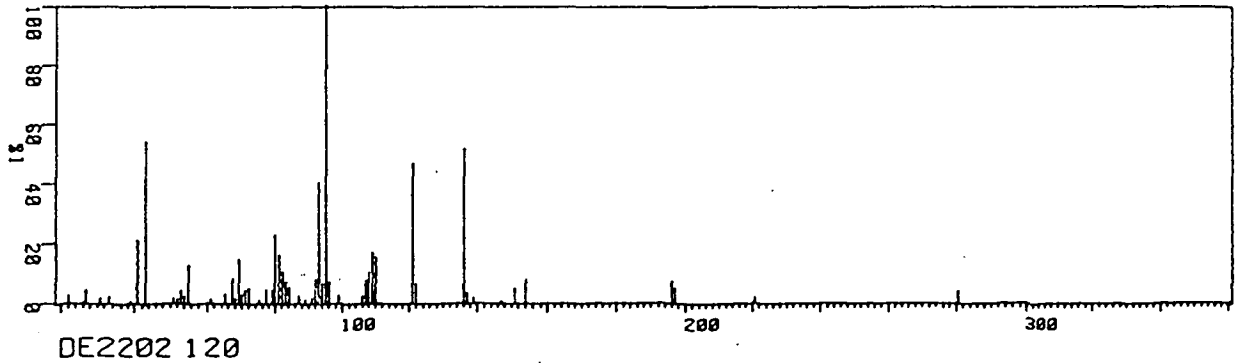

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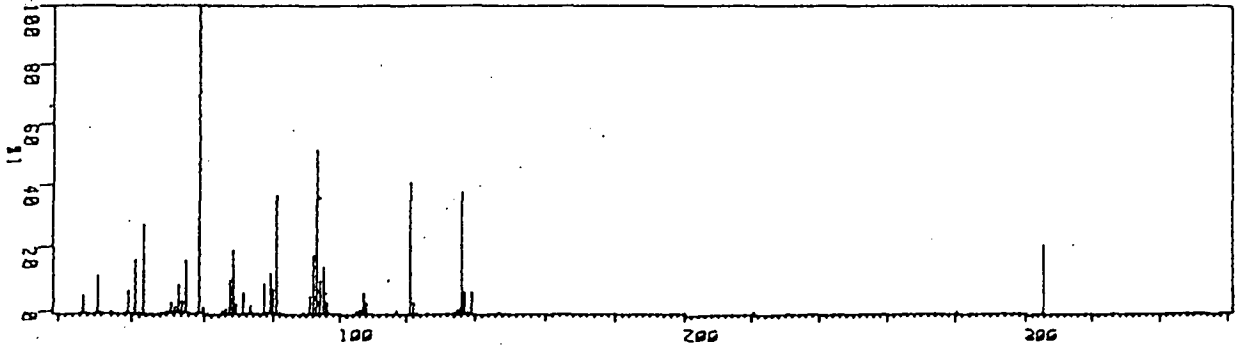

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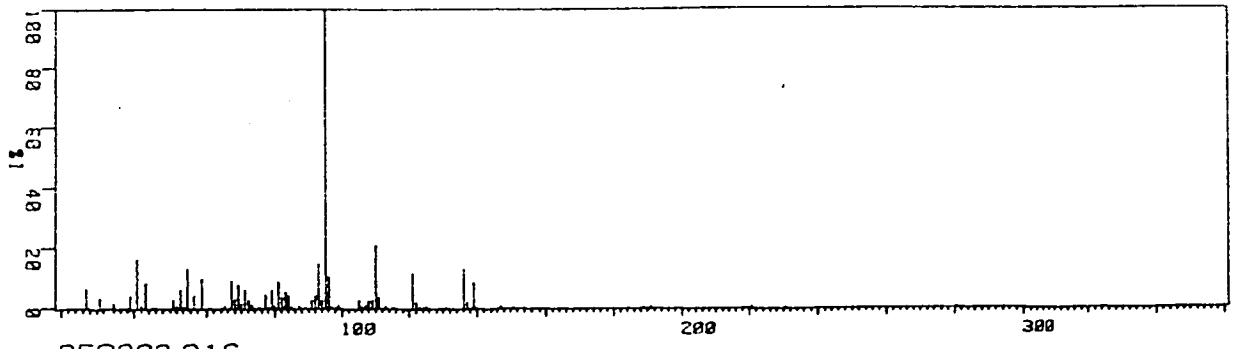

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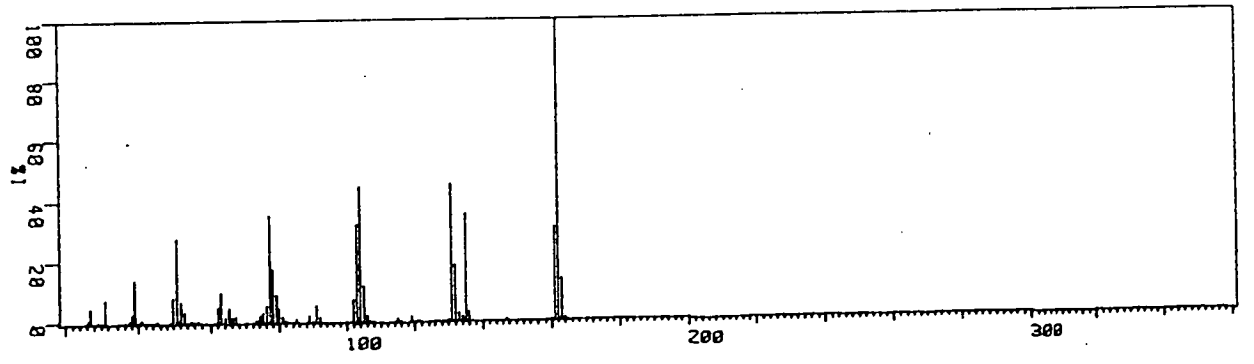

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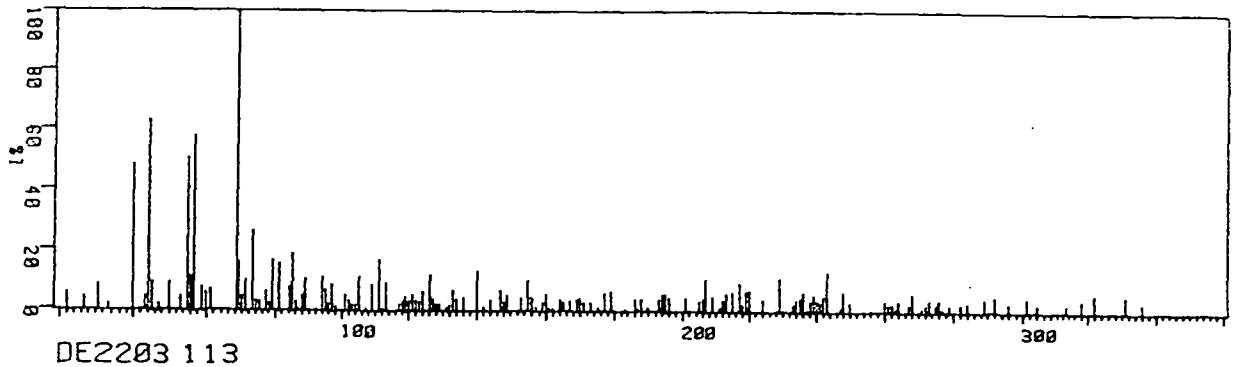

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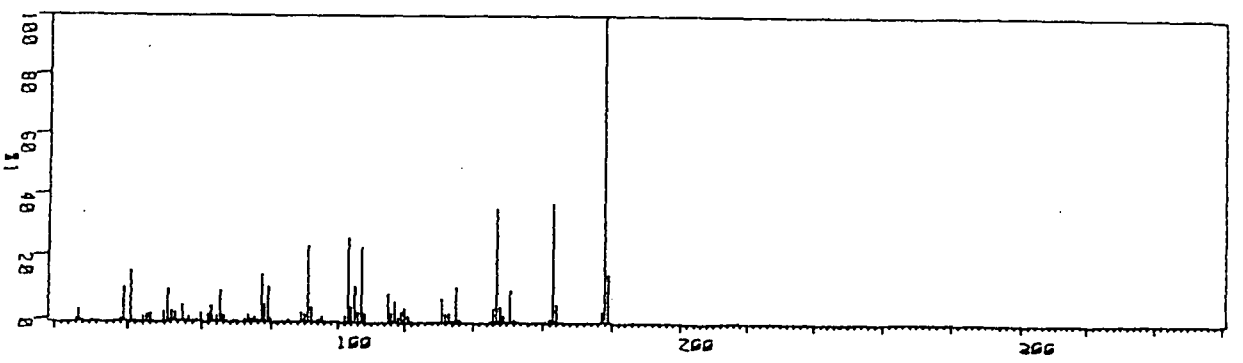

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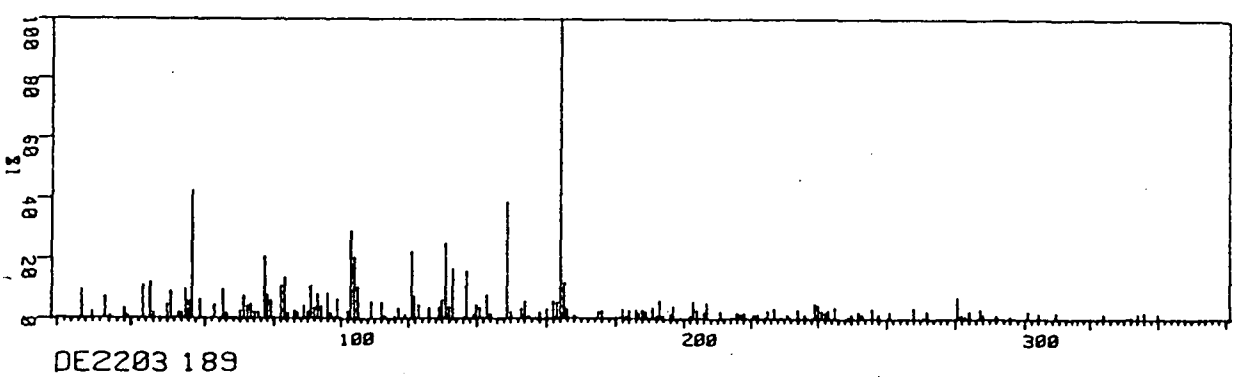
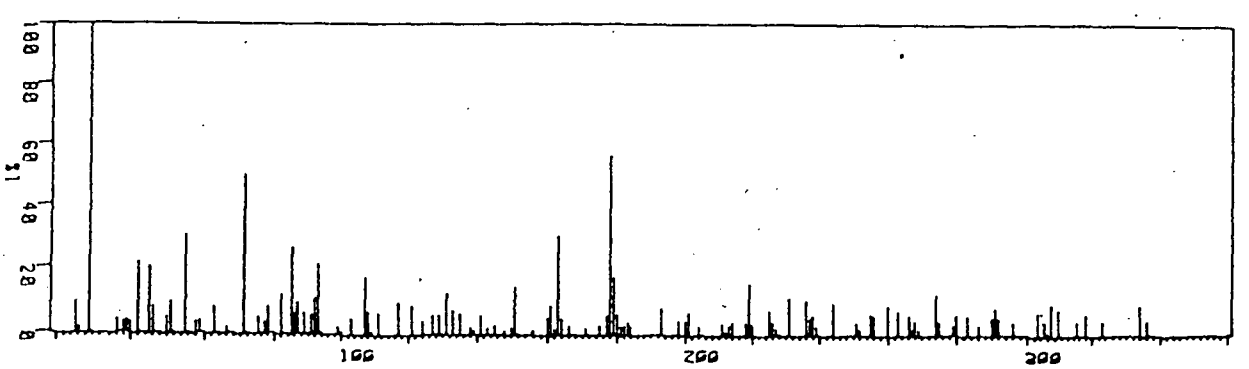


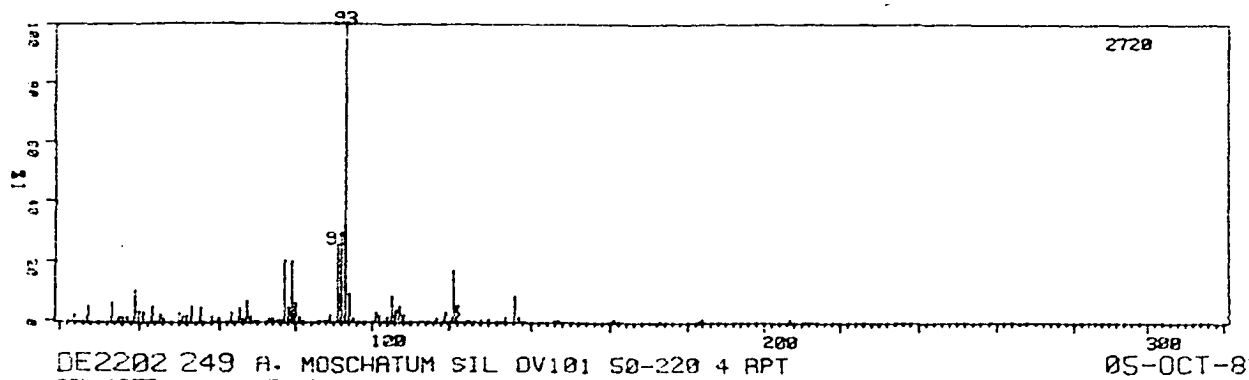
DE2203 189



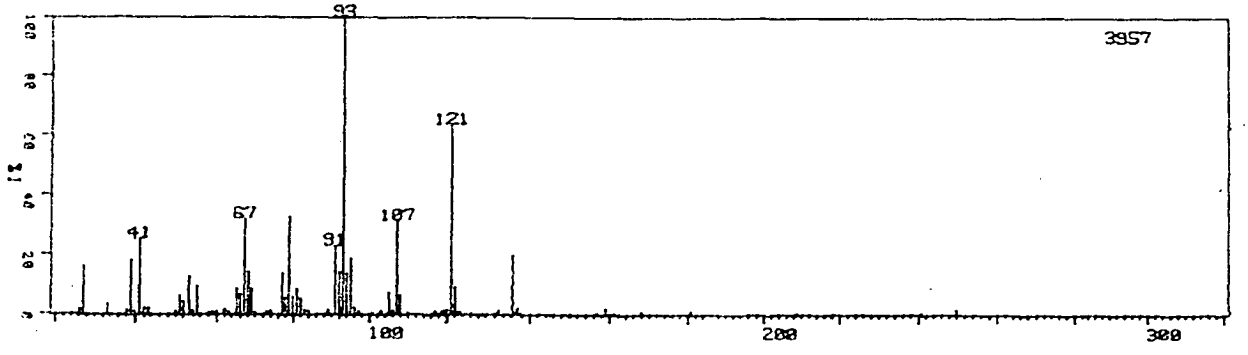
APPENDIX 3

Mass spectra of components of A. moschatum separated on OV-101 phase with reference to Total Ion Current trace Fig. 10.

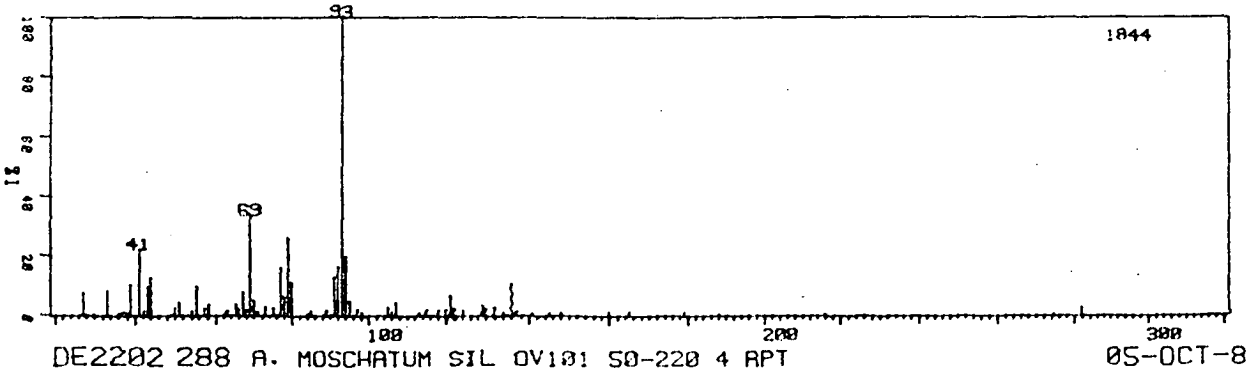
DE2202 237 A. MOSCHATUM SIL OV101 50-220 4 RPT 05-OCT-82
CAL:1D550 STA:E. 7:49



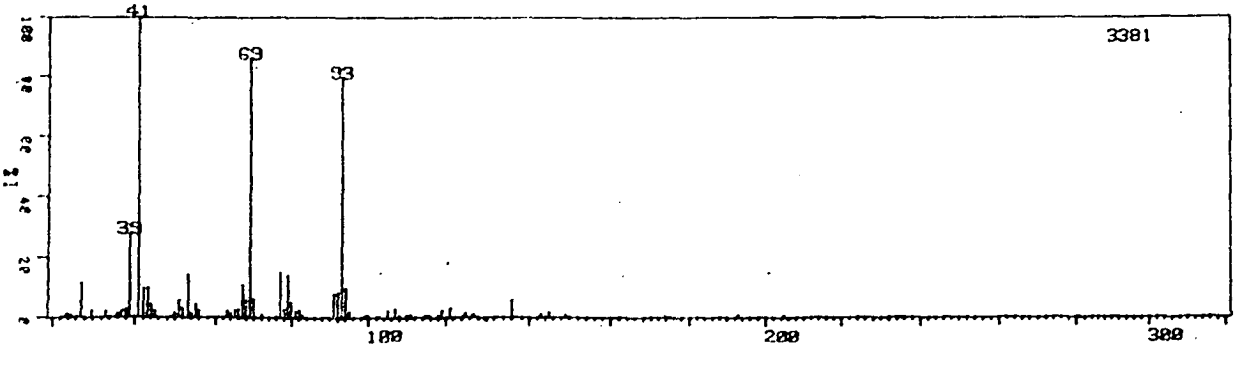
DE2202 249 A. MOSCHATUM SIL OV101 50-220 4 RPT 05-OCT-82
CAL:1D550 STA:E. 8:13

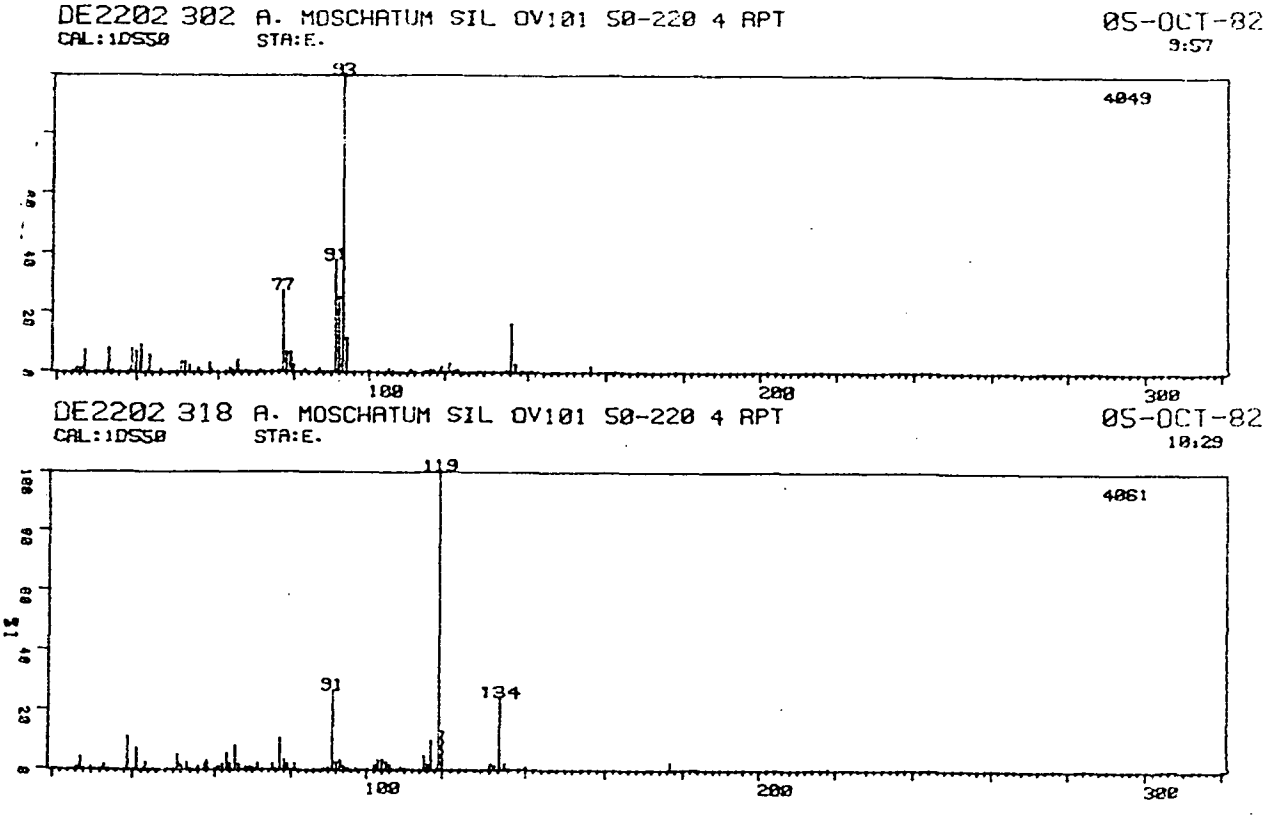


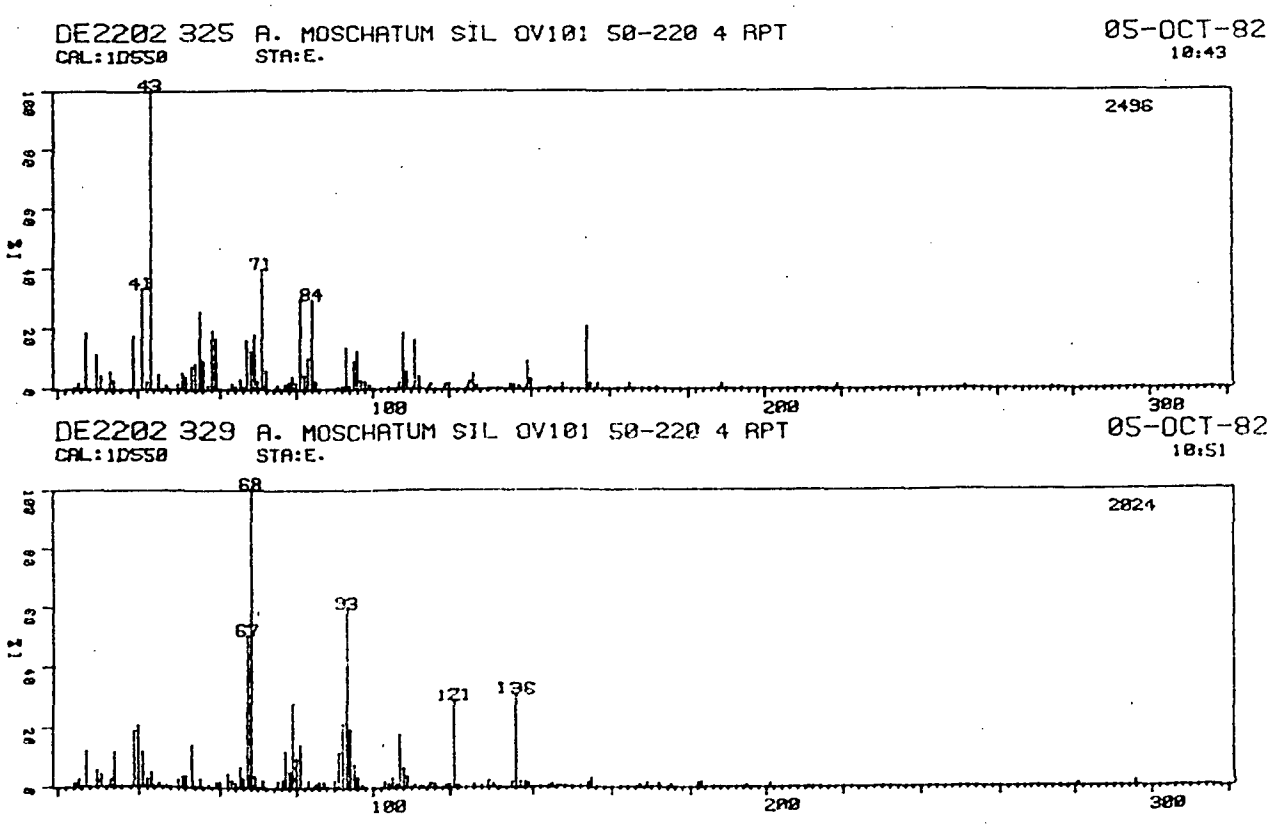
DE2202 275 A. MOSCHATUM SIL OV101 50-220 4 RPT 05-OCT-82
CAL:1D550 STA:E. 9:4



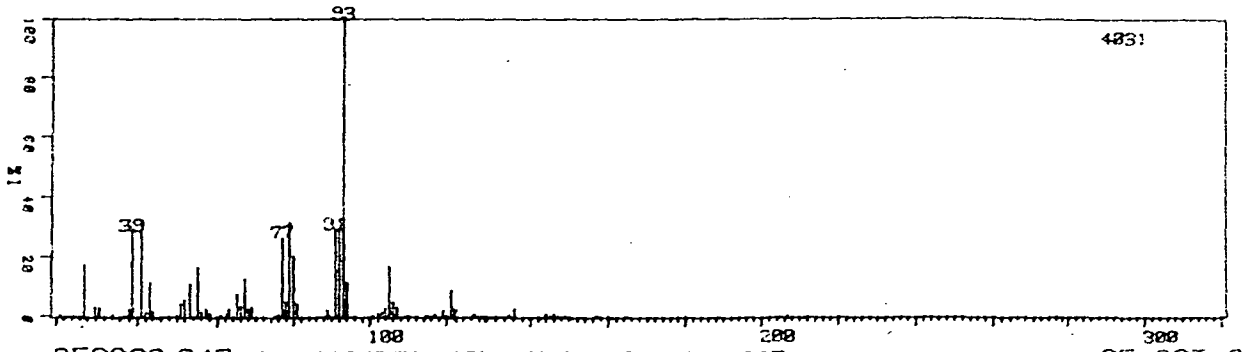
DE2202 288 A. MOSCHATUM SIL OV101 50-220 4 RPT 05-OCT-82
CAL:1D550 STA:E. 9:30



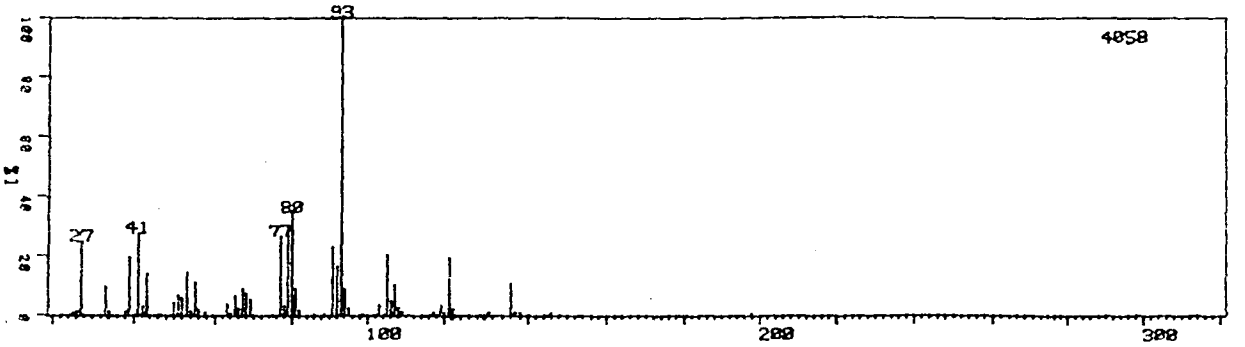

DE2202 302 A. MOSCHATUM SIL OV101 50-220 4 RPT
CAL:1DS50 STA:E.
05-OCT-82
9:57
4049
93
91
77
DE2202 318 A. MOSCHATUM SIL OV101 50-220 4 RPT
CAL:1DS50 STA:E.
05-OCT-82
10:29
4061
119
91
134
100
200
300


DE2202 325 A. MOSCHATUM SIL OV101 50-220 4 RPT
CAL:1DS50 STA:E.
05-OCT-82
10:43
2496
43
71
84
DE2202 329 A. MOSCHATUM SIL OV101 50-220 4 RPT
CAL:1DS50 STA:E.
05-OCT-82
10:51
2024
68
67
93
121
136
100
200
300

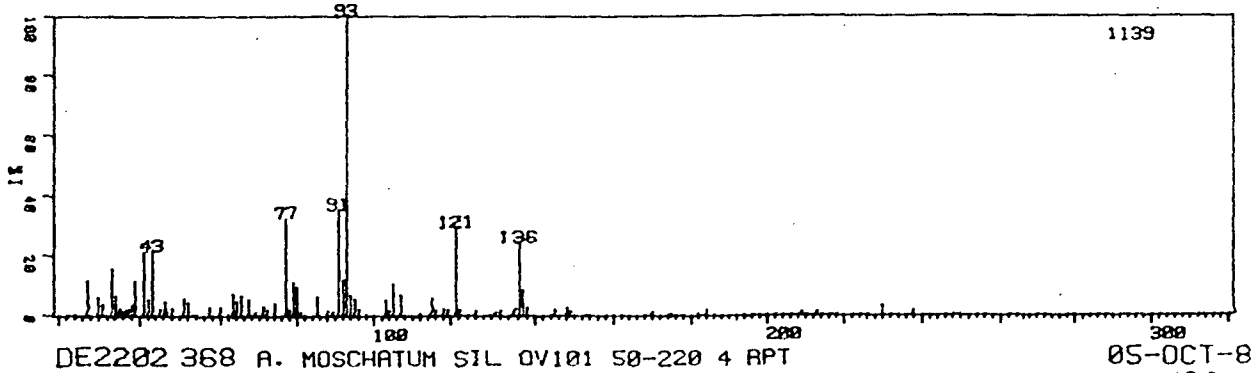
DE2202 335 A. MOSCHATUM SIL OV101 50-220 4 APT 05-OCT-82
CAL:1DSS0 STA:E. 11:2



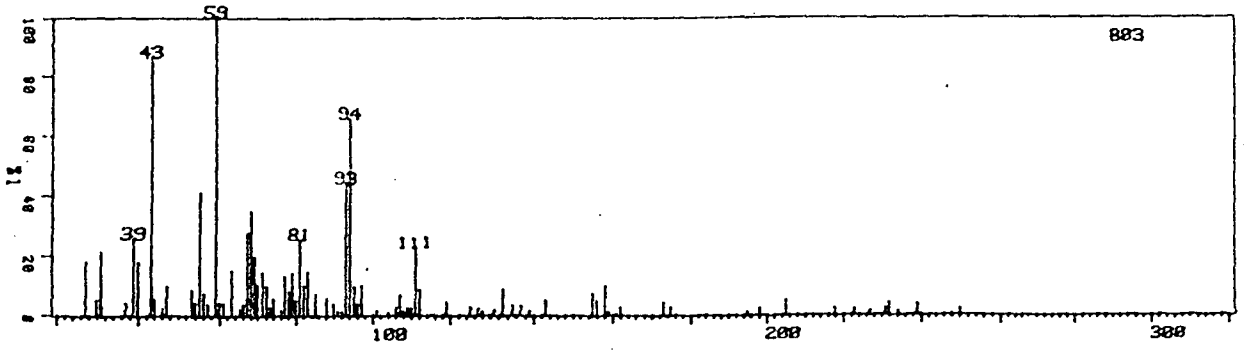
DE2202 347 A. MOSCHATUM SIL OV101 50-220 4 APT 05-OCT-82
CAL:1DSS0 STA:E. 11:26



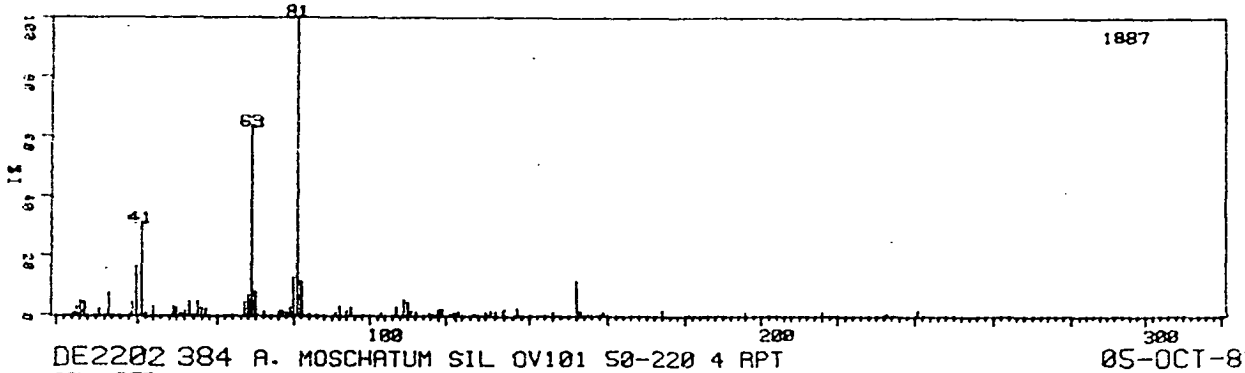
DE2202 358 A. MOSCHATUM SIL OV101 50-220 4 APT 05-OCT-82
CAL:1DSS0 STA:E. 11:48



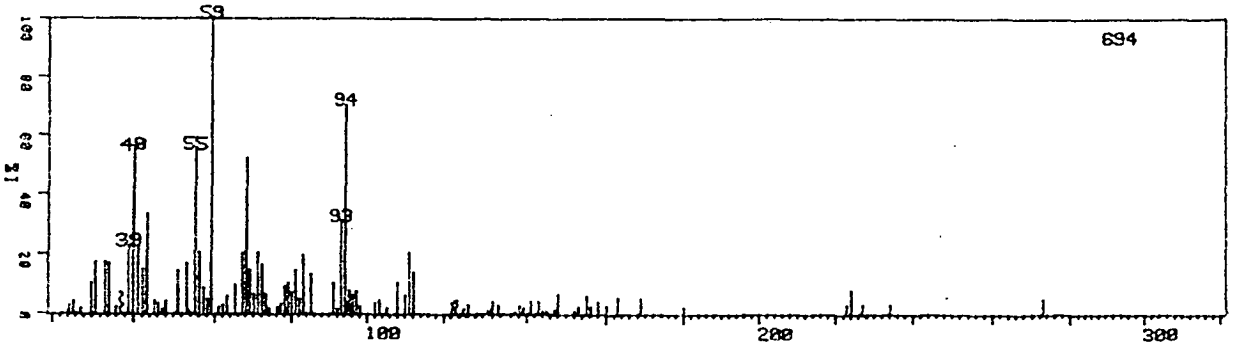
DE2202 368 A. MOSCHATUM SIL OV101 50-220 4 APT 05-OCT-82
CAL:1DSS0 STA:E. 12:8



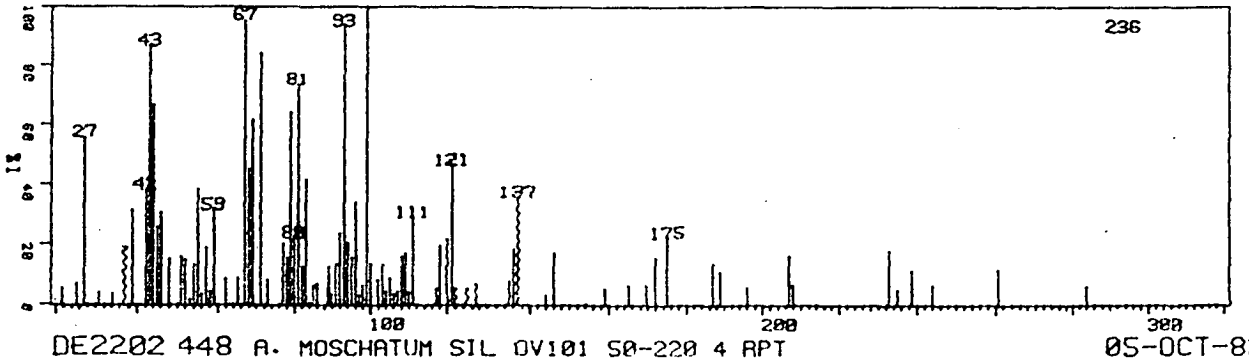
DE2202 379 A. MOSCHATUM SIL OV101 50-220 4 RPT 05-OCT-82
CAL:1DS50 STA:E. 12:29



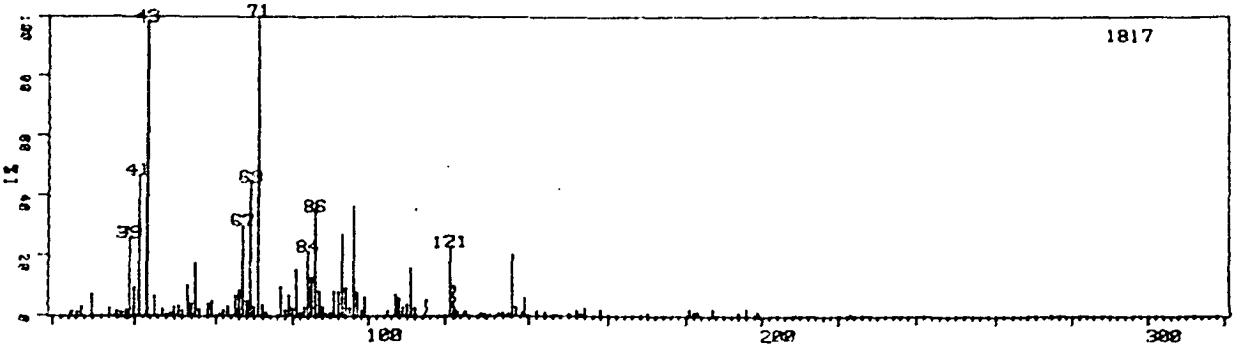
DE2202 384 A. MOSCHATUM SIL OV101 50-220 4 RPT 05-OCT-82
CAL:1DS50 STA:E. 12:39

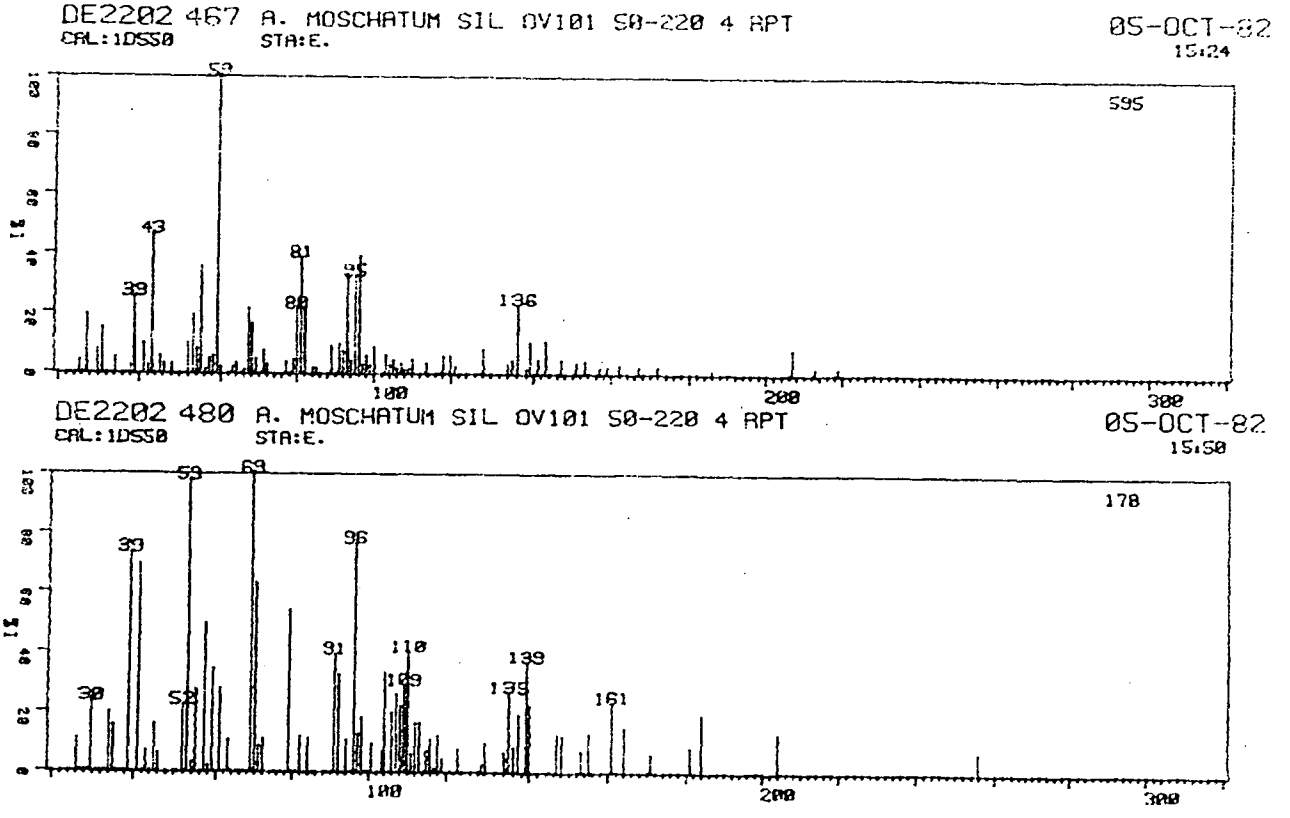


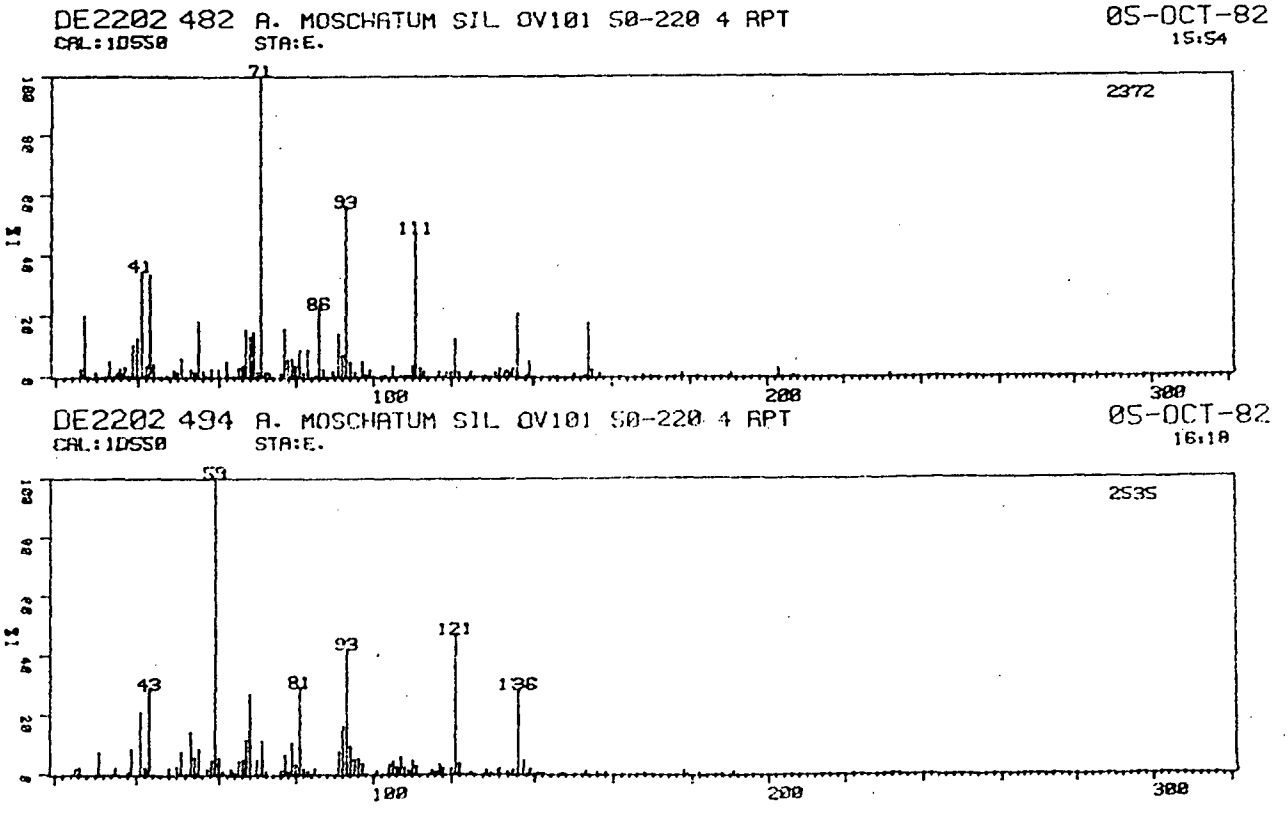
DE2202 421 A. MOSCHATUM SIL OV101 50-220 4 RPT 05-OCT-82
CAL:1DS50 STA:E. 13:53

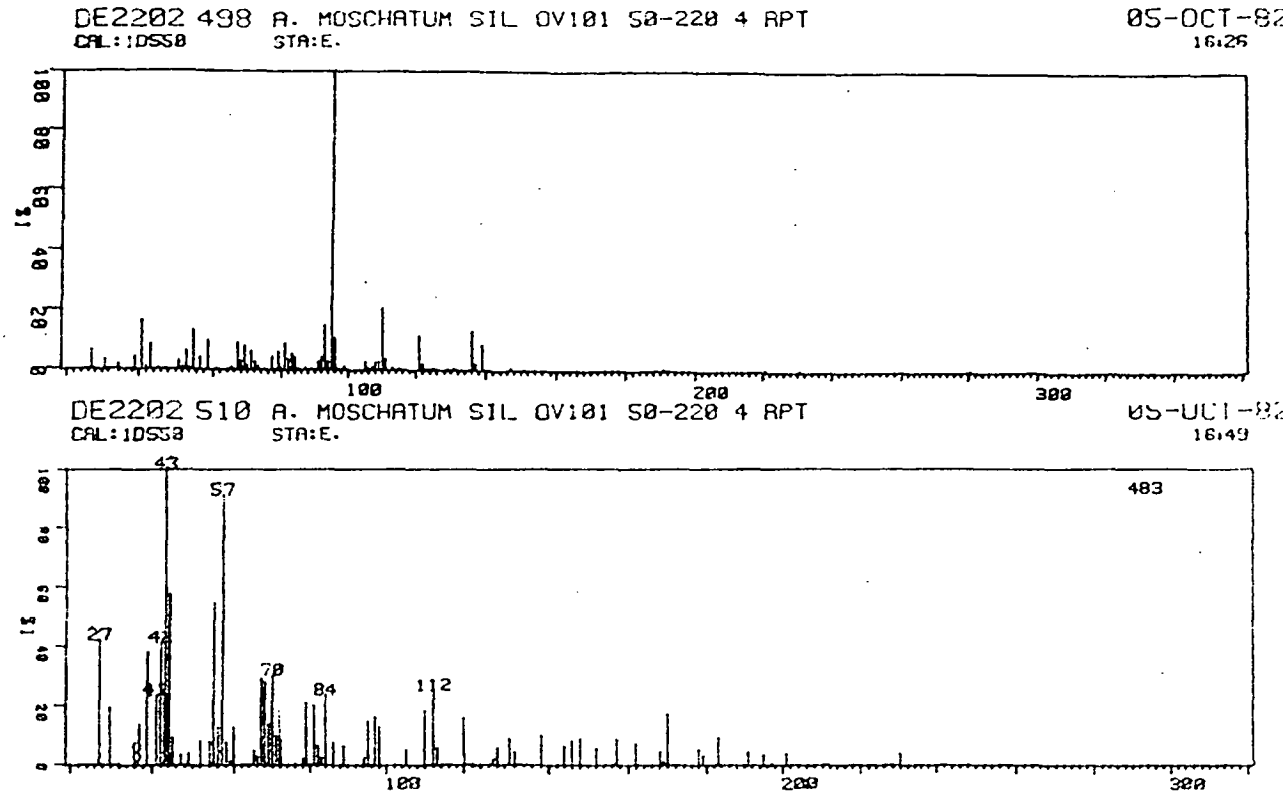


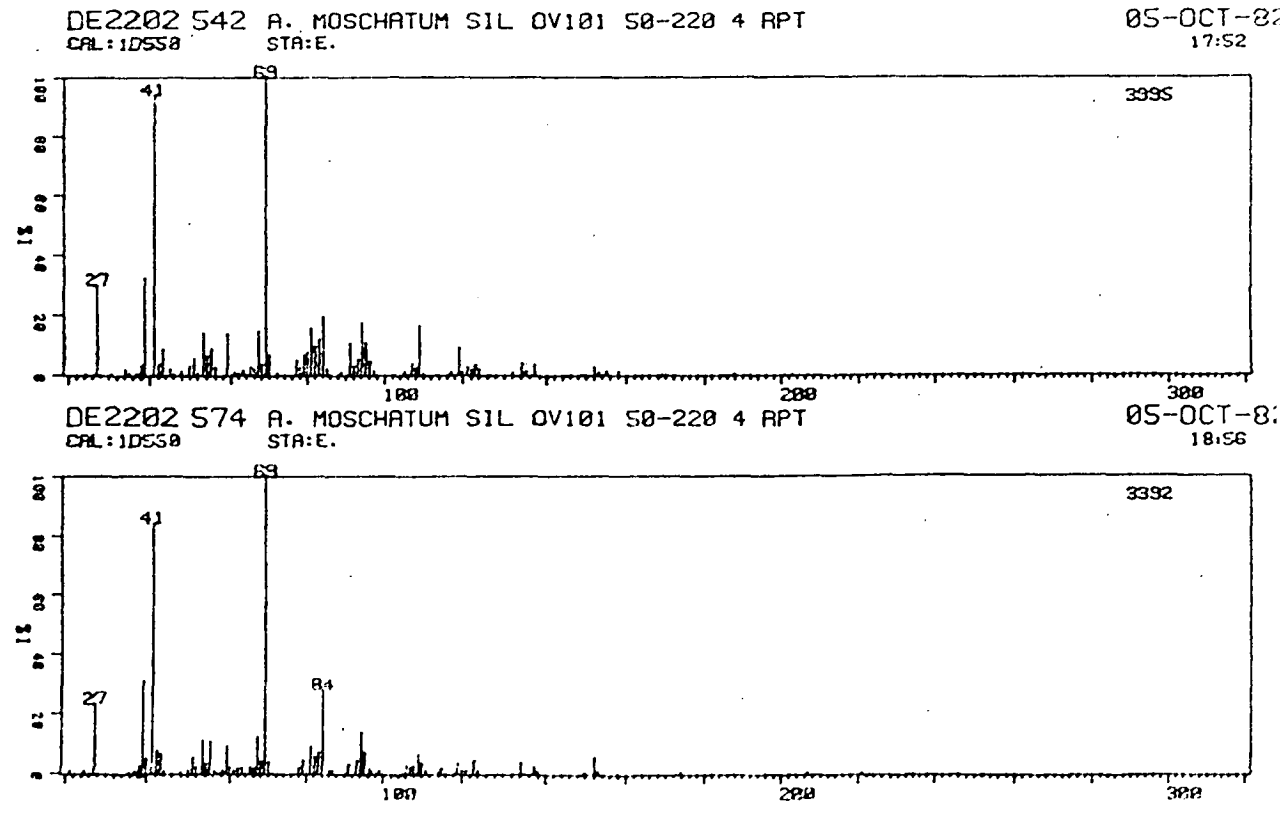
DE2202 448 A. MOSCHATUM SIL OV101 50-220 4 RPT 05-OCT-82
CAL:1DS50 STA:E. 14:47

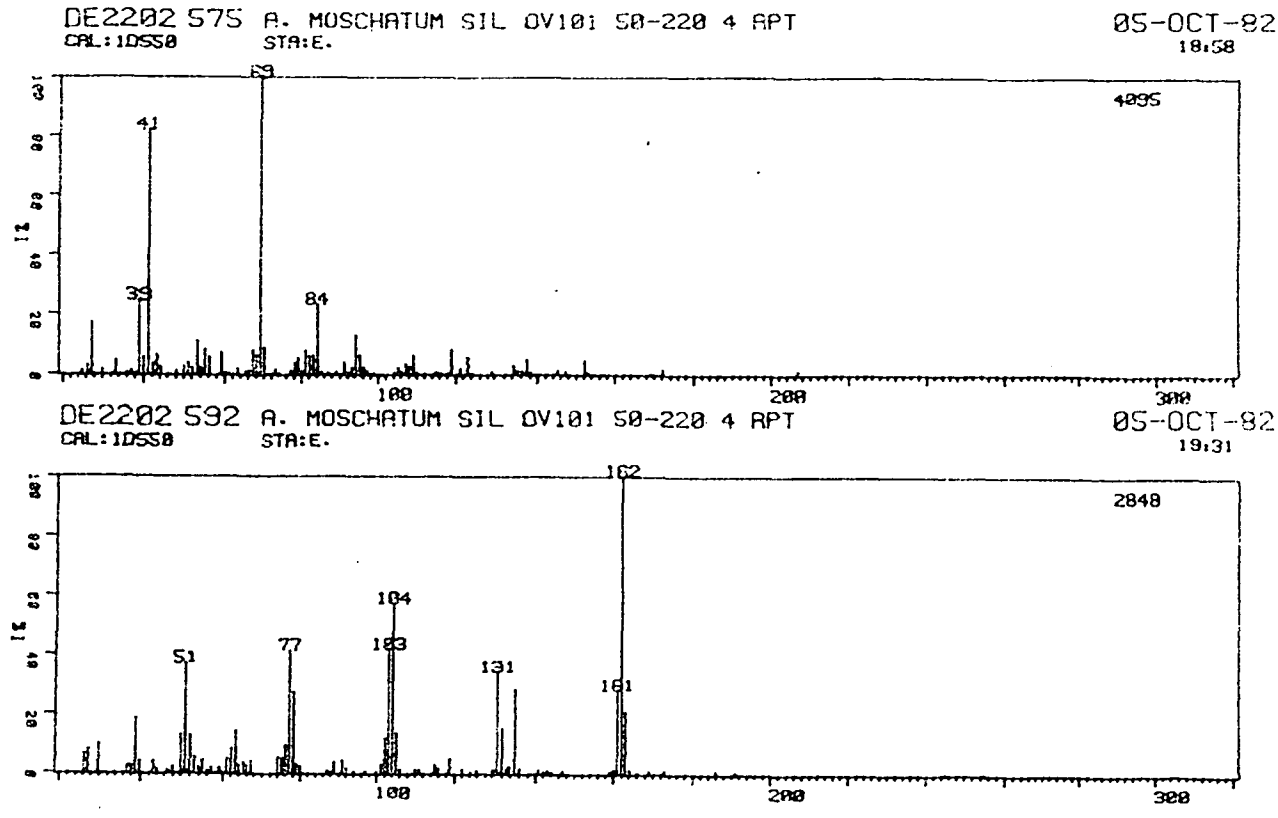


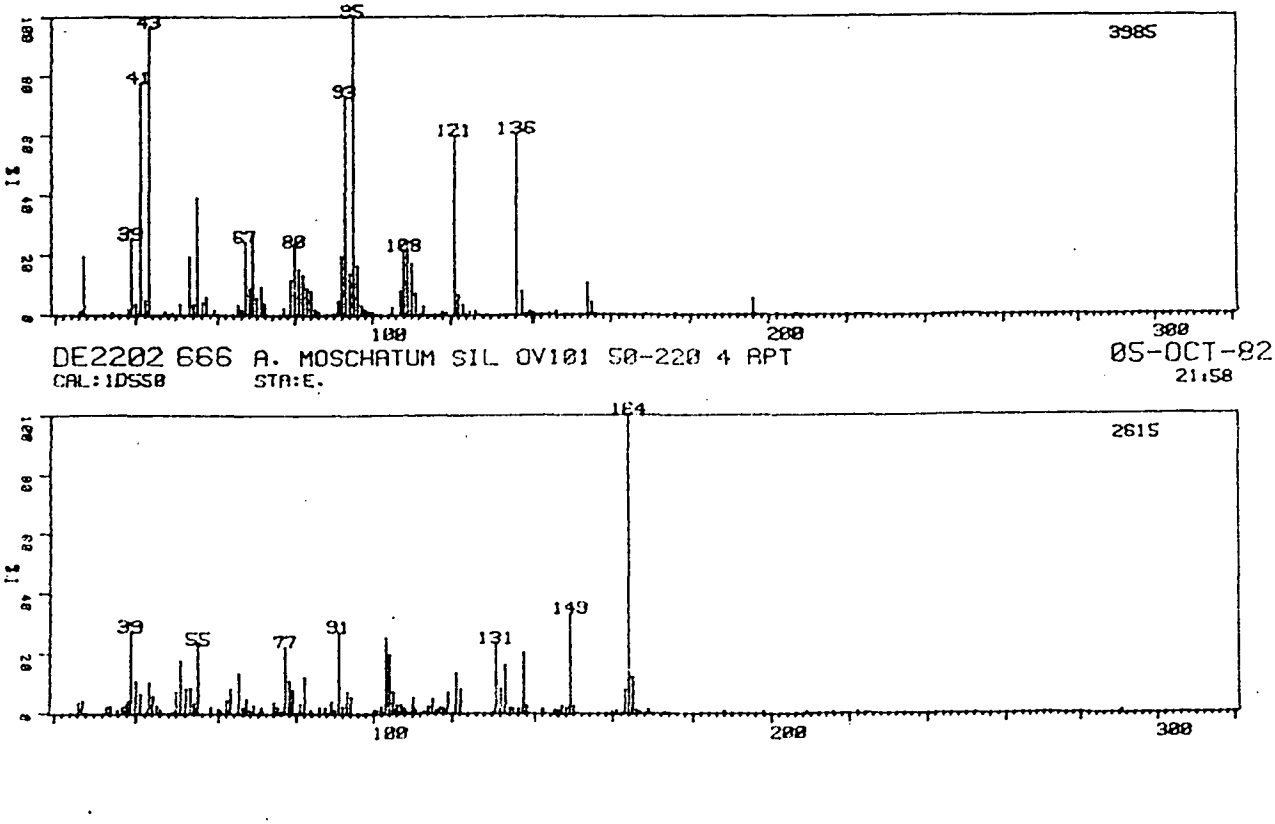

DE2202 467 A. MOSCHATUM SIL OV101 S0-220 4 RPT
CAL:1DSS0 STA:E.
05-OCT-82
15:24
595
DE2202 480 A. MOSCHATUM SIL OV101 S0-220 4 RPT
CAL:1DSS0 STA:E.
05-OCT-82
15:50
178


DE2202 482 A. MOSCHATUM SIL OV101 S0-220 4 RPT
CAL:1DSS0 STA:E.
05-OCT-82
15:54
2372
DE2202 494 A. MOSCHATUM SIL OV101 S0-220 4 RPT
CAL:1DSS0 STA:E.
05-OCT-82
16:18
2535


DE2202 498 A. MOSCHATUM SIL OV101 50-220 4 RPT
CAL:1DS50 STA:E.
05-OCT-82
16:26
DE2202 510 A. MOSCHATUM SIL OV101 50-220 4 RPT
CAL:1DS50 STA:E.
16:49
43
57
27
41
70
84
112
483
100
200
300

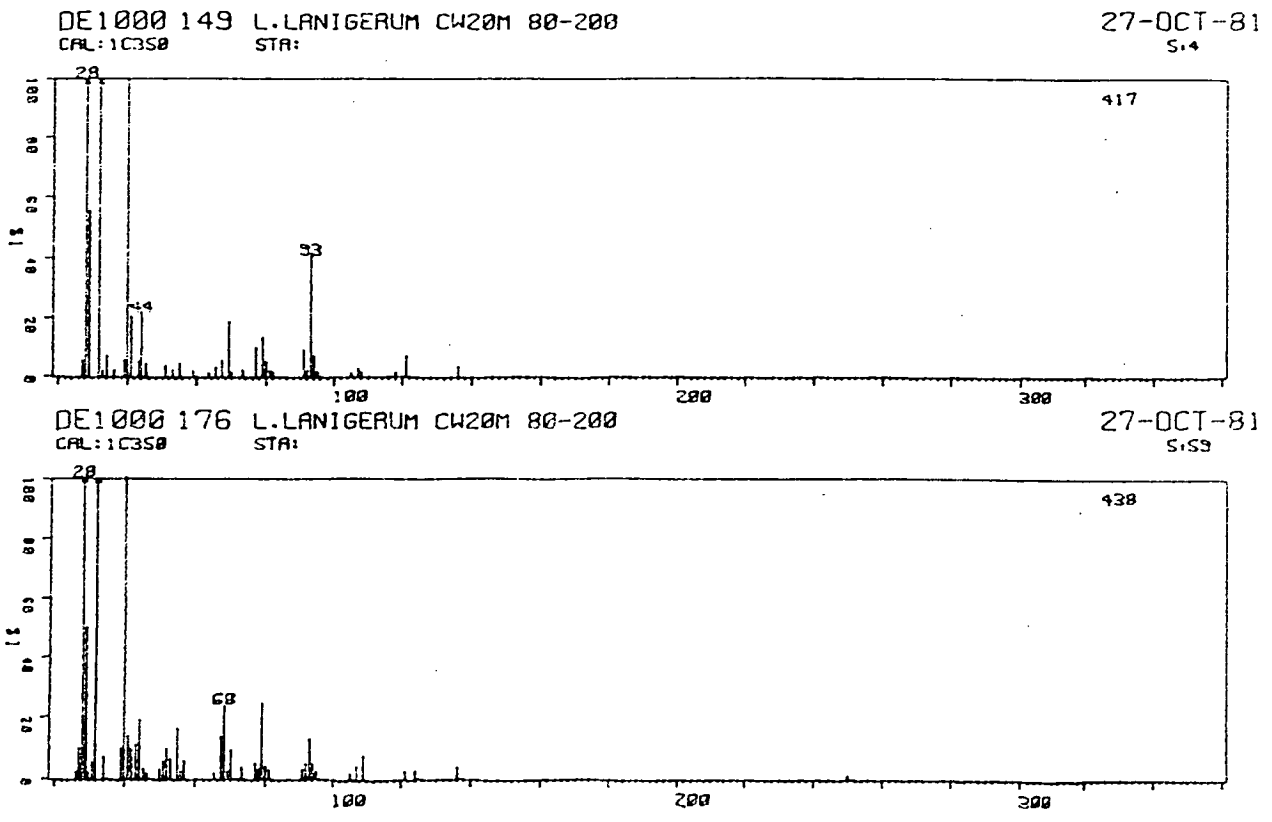

DE2202 542 A. MOSCHATUM SIL OV101 50-220 4 RPT
CAL:1DS50 STA:E.
05-OCT-82
17:52
69
41
27
3395
DE2202 574 A. MOSCHATUM SIL OV101 50-220 4 RPT
CAL:1DS50 STA:E.
18:56
69
41
27
84
3392
100
200
300

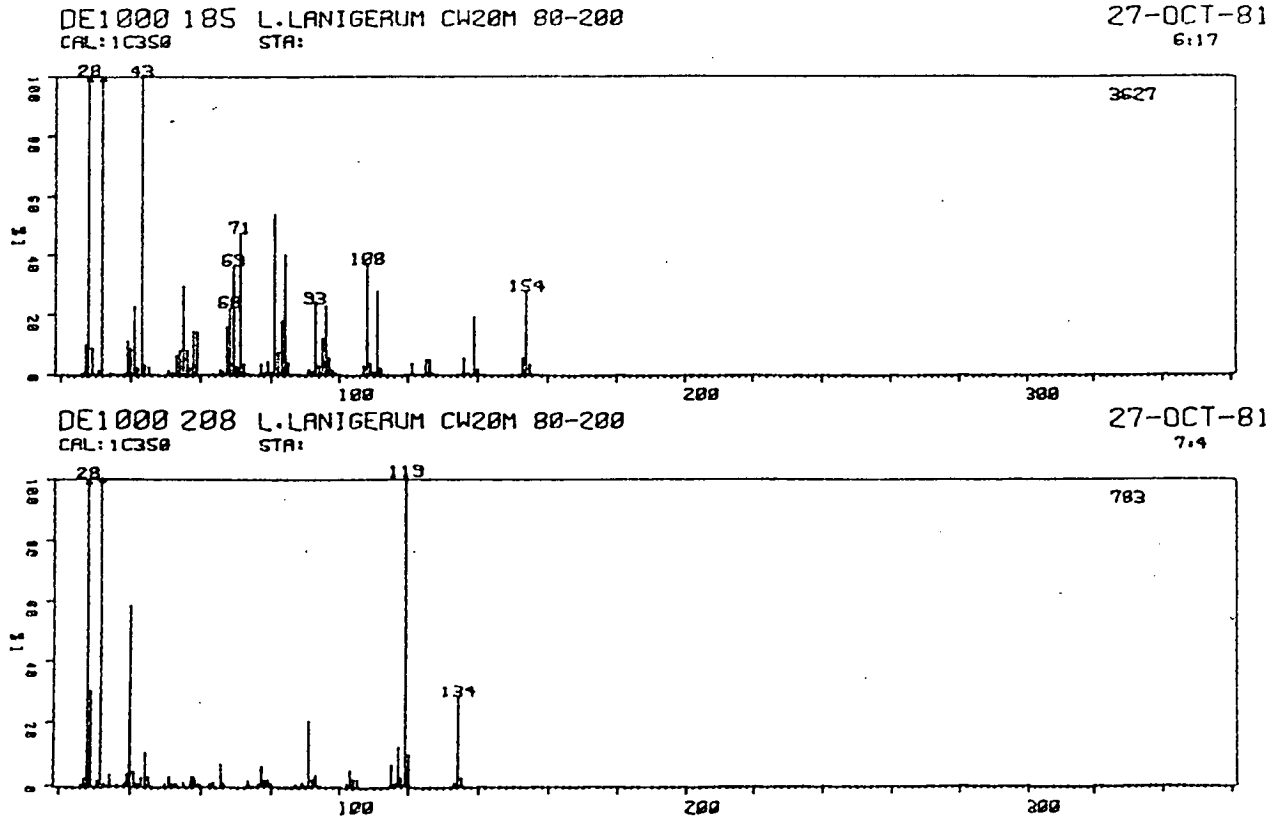

DE2202 575 A. MOSCHATUM SIL OV101 50-220 4 RPT
05-OCT-82
CAL:1DSS0
STA:E.
18:58
4095
69
41
39
84
100
200
300
DE2202 592 A. MOSCHATUM SIL OV101 50-220 4 RPT
05-OCT-82
CAL:1DSS0
STA:E.
19:31
2848
162
104
103
77
51
131
161
100
200
300


DE2202 602 A. MOSCHATUM SIL OV101 50-220 4 RPT
05-OCT-82
CAL:1DSS0
STA:E.
19:52
3985
95
43
41
93
121
136
39
67
80
108
100
200
300
DE2202 666 A. MOSCHATUM SIL OV101 50-220 4 RPT
05-OCT-82
CAL:1DSS0
STA:E.
21:58
2615
164
149
39
55
77
91
131
100
200
300

APPENDIX 4

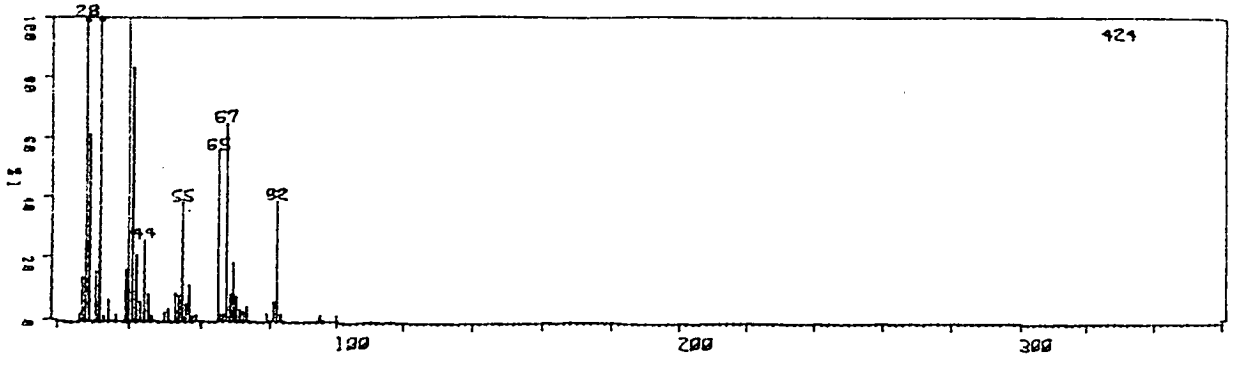
Mass spectra of components of L. lanigerum separated on CW-20m phase with reference to total ion current trace Fig. 11.


DE1000 149 L.LANIGERUM CW20M 80-200
CAL:1C3S0 STA:
27-OCT-81
S:4
28
417
93
14
100
200
300
DE1000 176 L.LANIGERUM CW20M 80-200
CAL:1C3S0 STA:
27-OCT-81
S:59
28
438
68
100
200
300

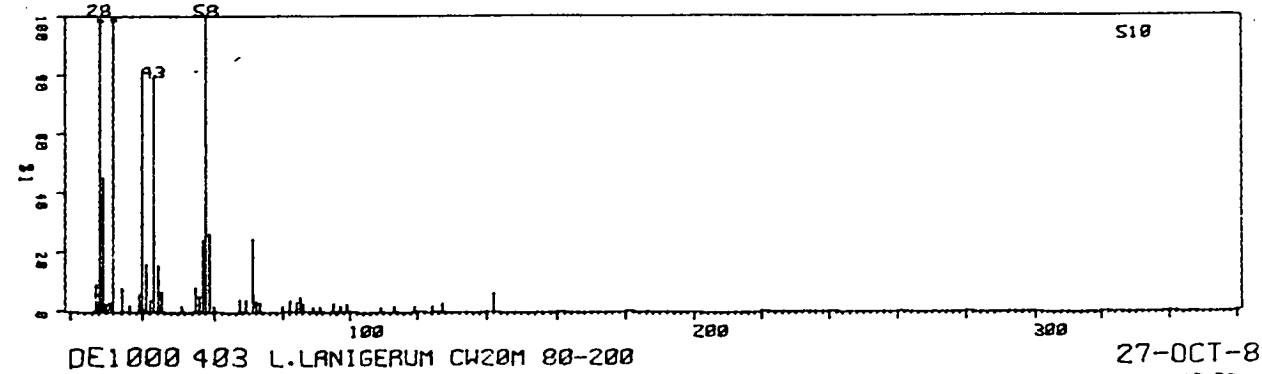

DE1000 185 L.LANIGERUM CW20M 80-200
CAL:1C3S0 STA:
27-OCT-81
6:17
28
43
3627
71
69
68
93
108
154
100
200
300
DE1000 208 L.LANIGERUM CW20M 80-200
CAL:1C3S0 STA:
27-OCT-81
7:4
28
119
783
134
100
200
300

DE1000 258 L.LANIGERUM CW20M 80-200 27-OCT-81
CAL:1C3S0 STA: 8:45

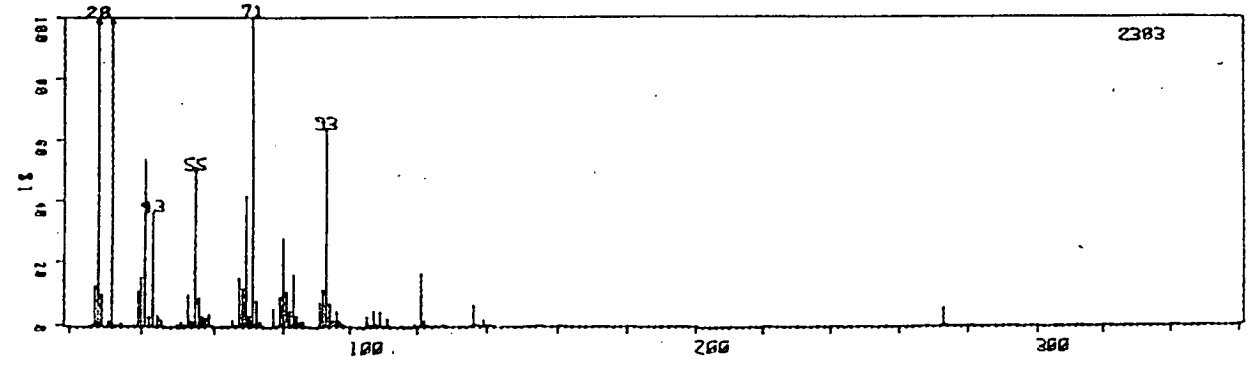
DE1000 275 L.LANIGERUM CW20M 80-200 27-OCT-81
CAL:1C3S0 STA: 9:20



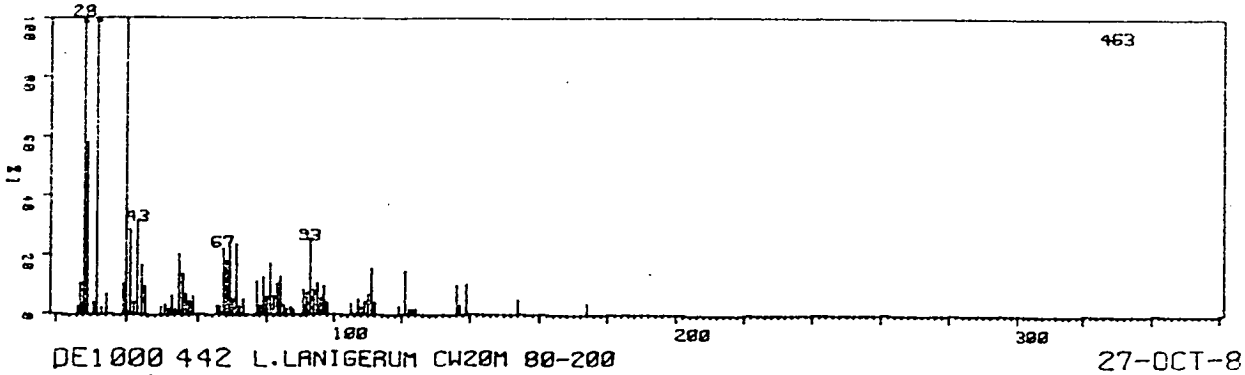
DE1000 281 L.LANIGERUM CW20M 80-200 27-OCT-81
CAL:1C3S0 STA: 9:32



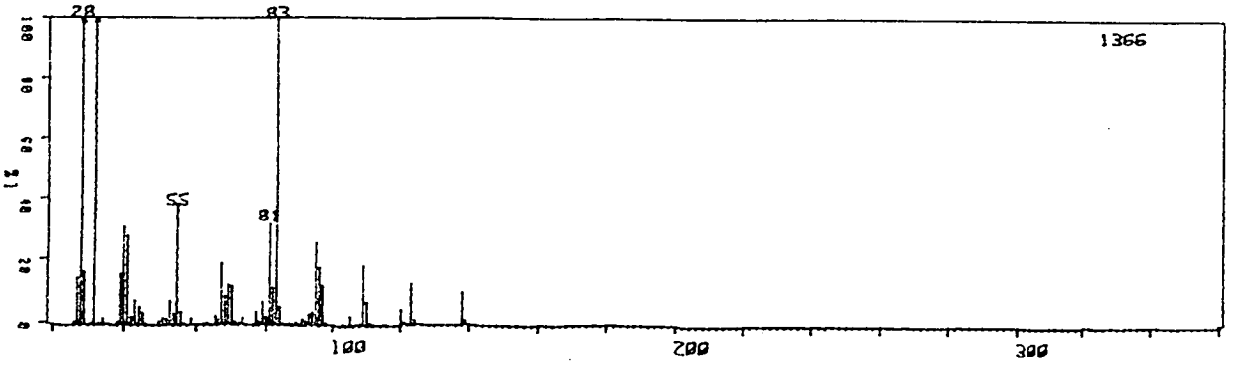
DE1000 403 L.LANIGERUM CW20M 80-200 27-OCT-81
CAL:1C3S0 STA: 13:39



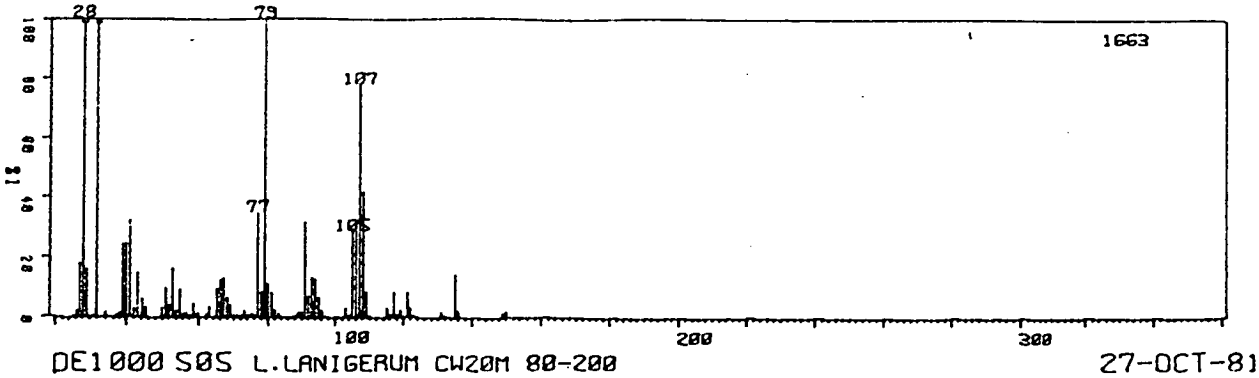
DE1000 420 L.LANIGERUM CW20M 80-200 27-OCT-81
CAL:1C3S0 STA: 14:14



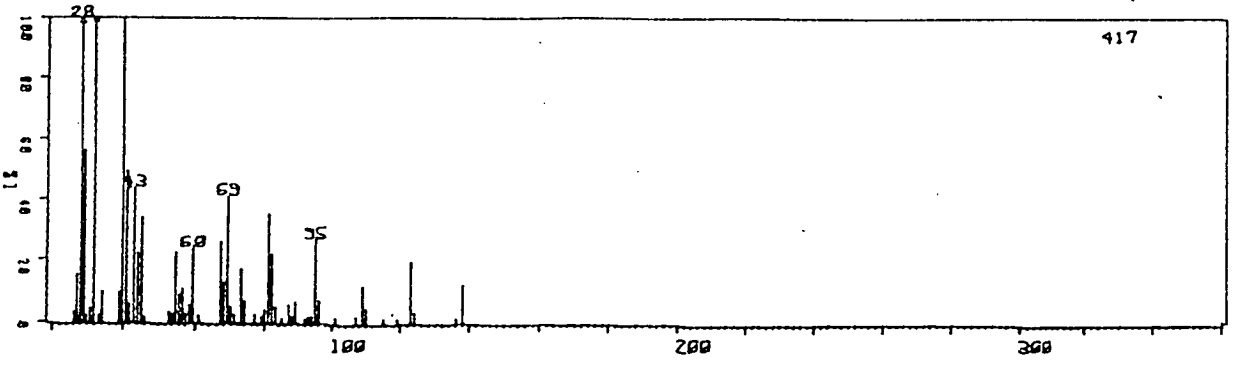
DE1000 442 L.LANIGERUM CW20M 80-200 27-OCT-81
CAL:1C3S0 STA: 14:58



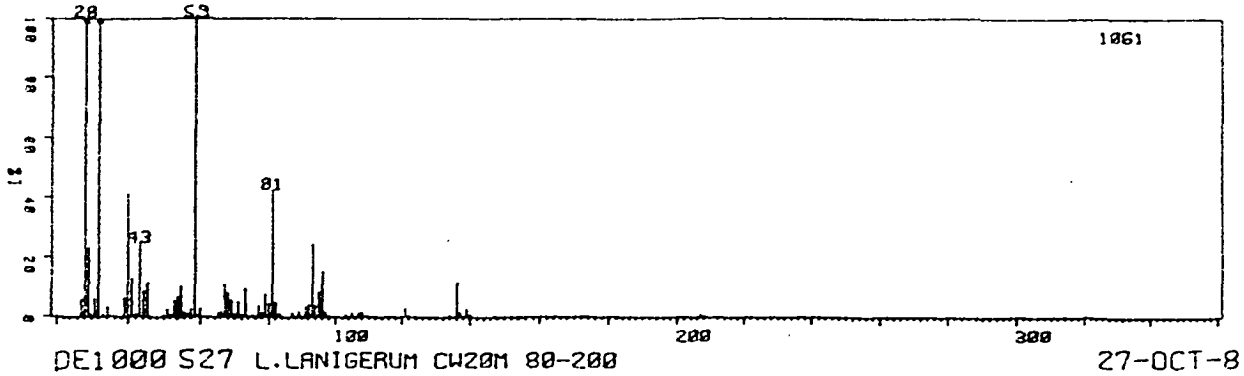
DE1000 479 L.LANIGERUM CW20M 80-200 27-OCT-81
CAL:1C3S0 STA: 16:13



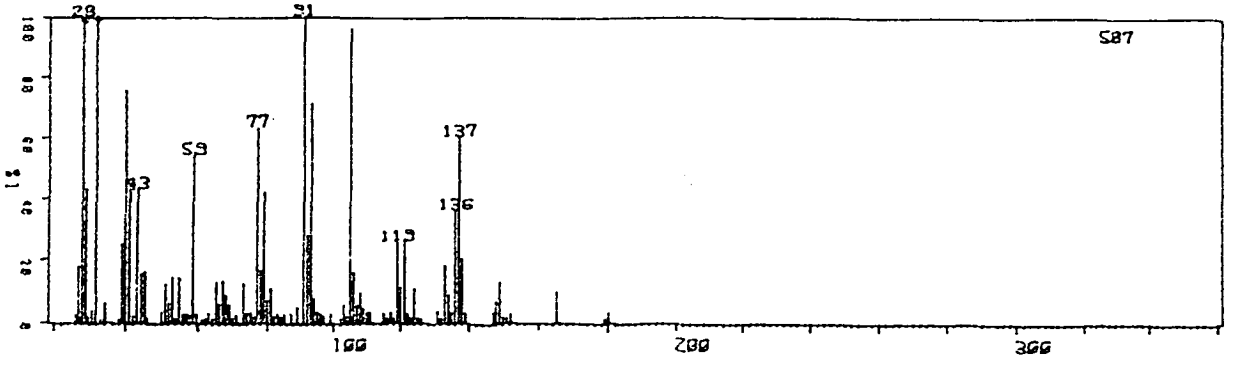
DE1000 505 L.LANIGERUM CW20M 80-200 27-OCT-81
CAL:1C3S0 STA: 17:6



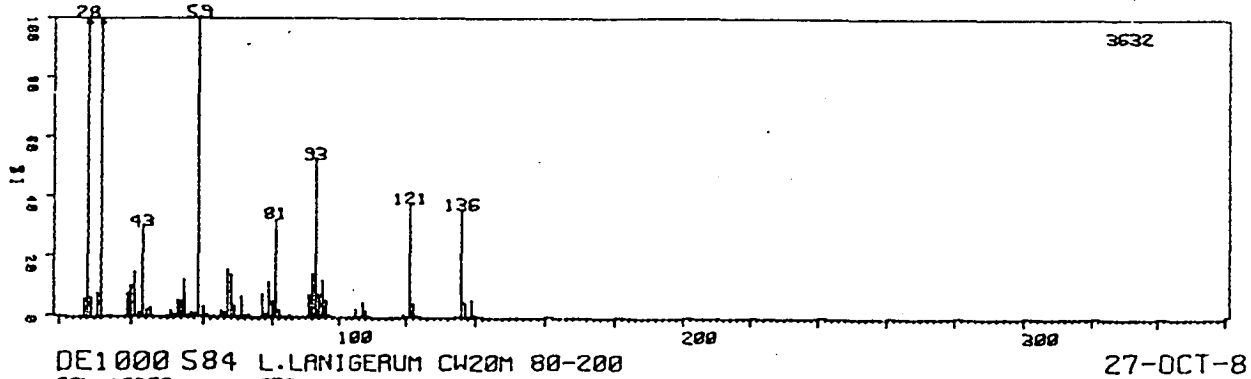
DE1000 S12 L.LANIGERUM CW20M 80-200 27-OCT-81
CAL: 1C3S0 STA: 17:20



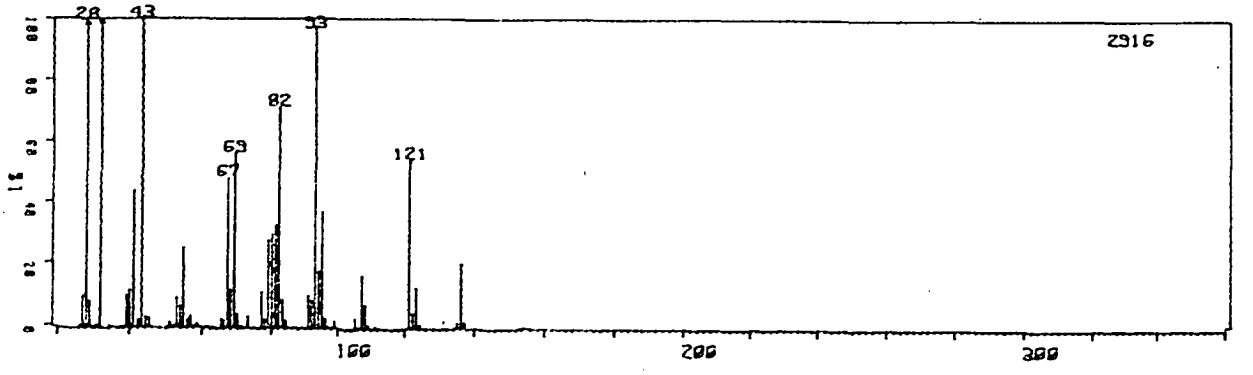
DE1000 S27 L.LANIGERUM CW20M 80-200 27-OCT-81
CAL: 1C3S0 STA: 17:51



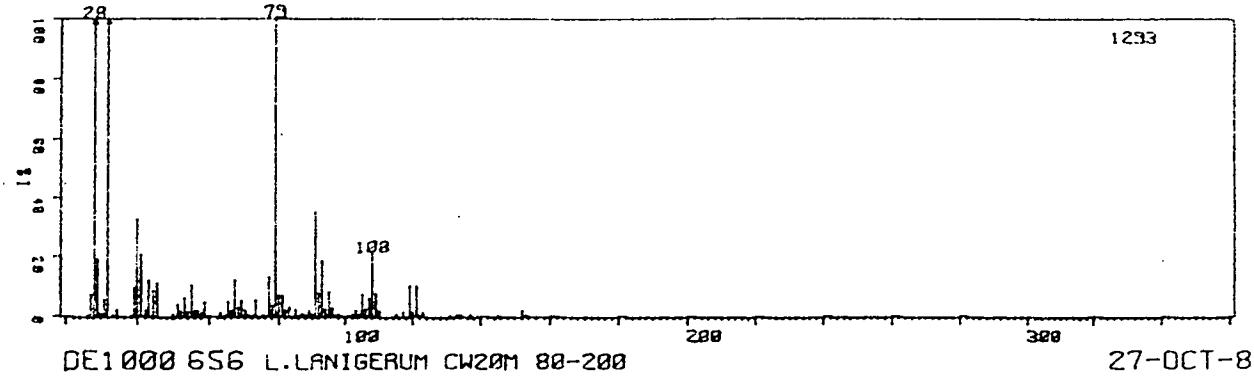
DE1000 S34 L.LANIGERUM CW20M 80-200 27-OCT-81
CAL: 1C3S0 STA: 18:5



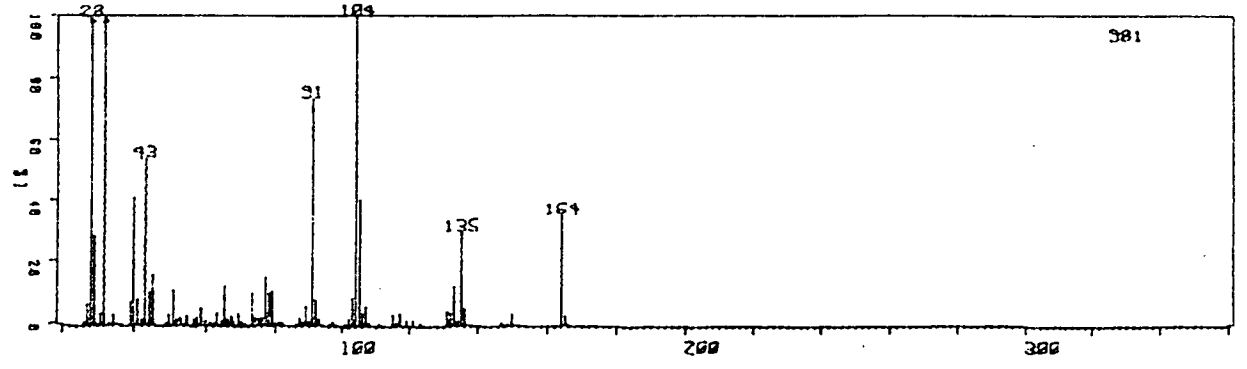
DE1000 S84 L.LANIGERUM CW20M 80-200 27-OCT-81
CAL: 1C3S0 STA: 19:46



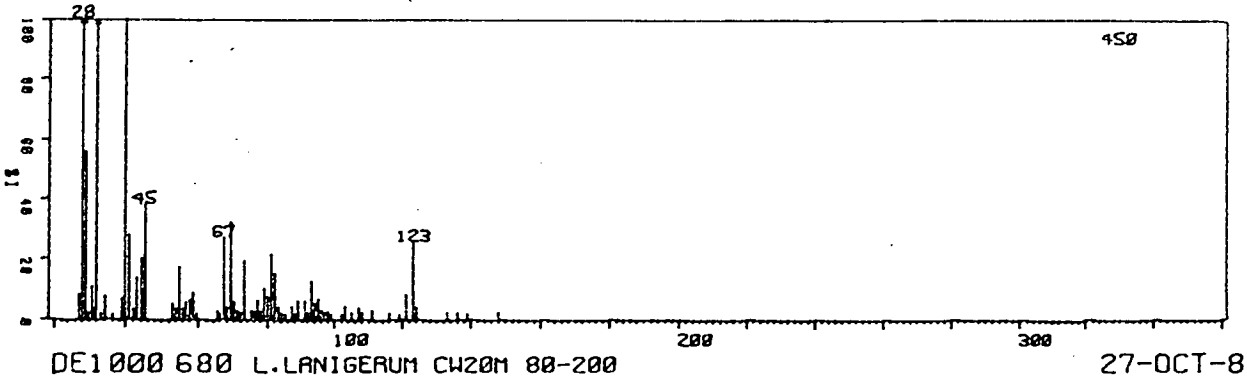
DE1000 614 L.LANIGERUM CW20M 80-200 27-OCT-81
CAL: 1C3S0 STA: 20:47



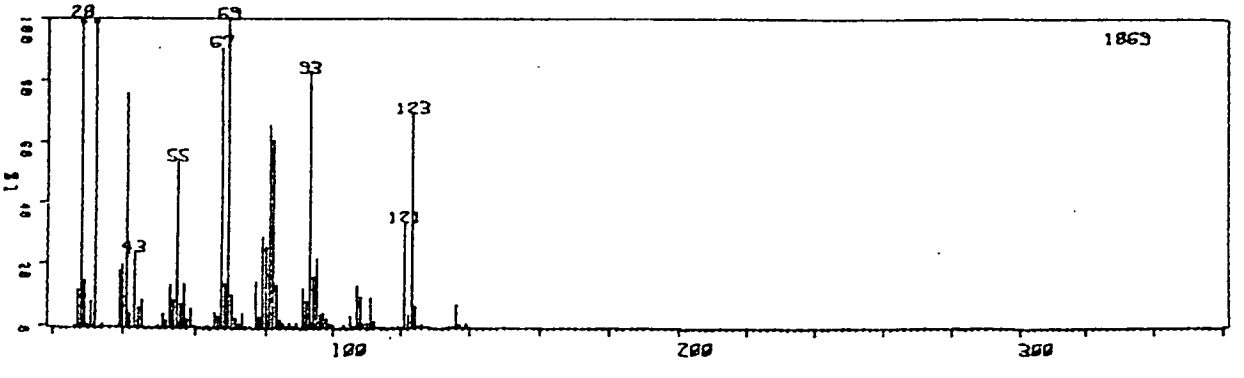
DE1000 656 L.LANIGERUM CW20M 80-200 27-OCT-81
CAL: 1C3S0 STA: 22:13



DE1000 668 L.LANIGERUM CW20M 80-200 27-OCT-81
CAL: 1C3S0 STA: 22:37



DE1000 680 L.LANIGERUM CW20M 80-200 27-OCT-81
CAL: 1C3S0 STA: 23:1



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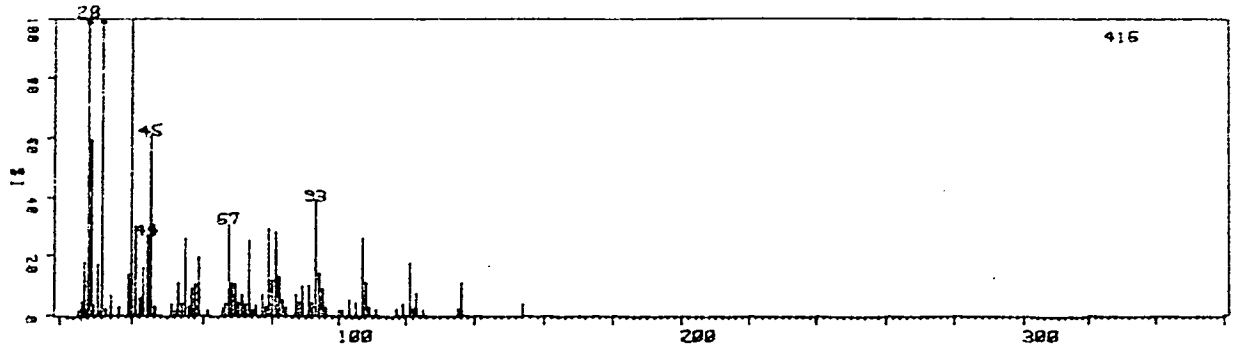

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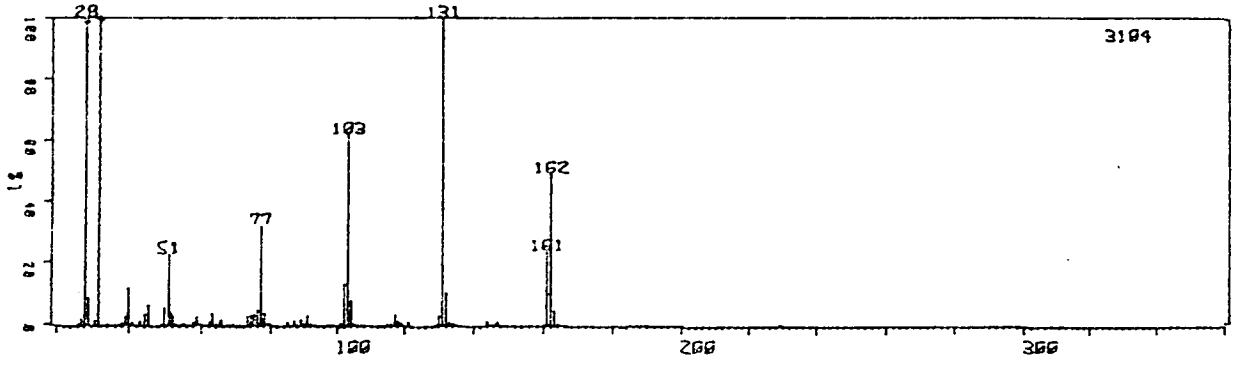

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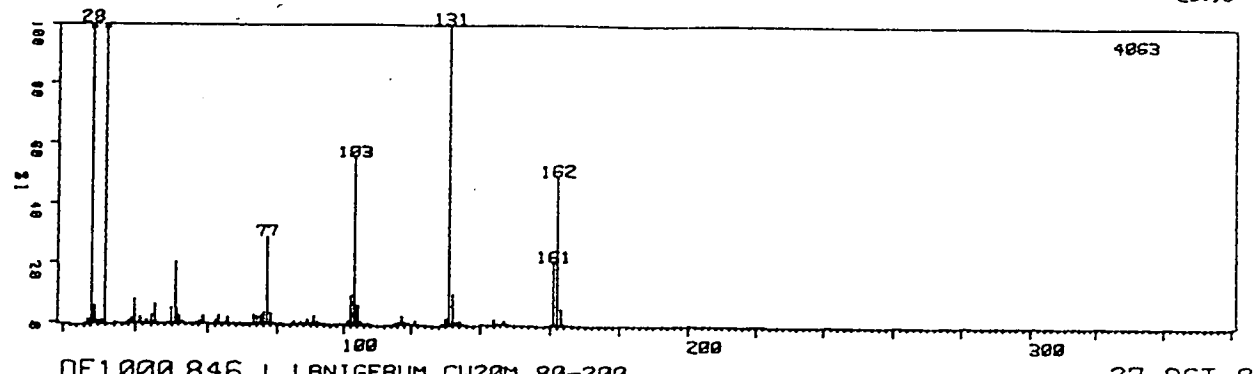

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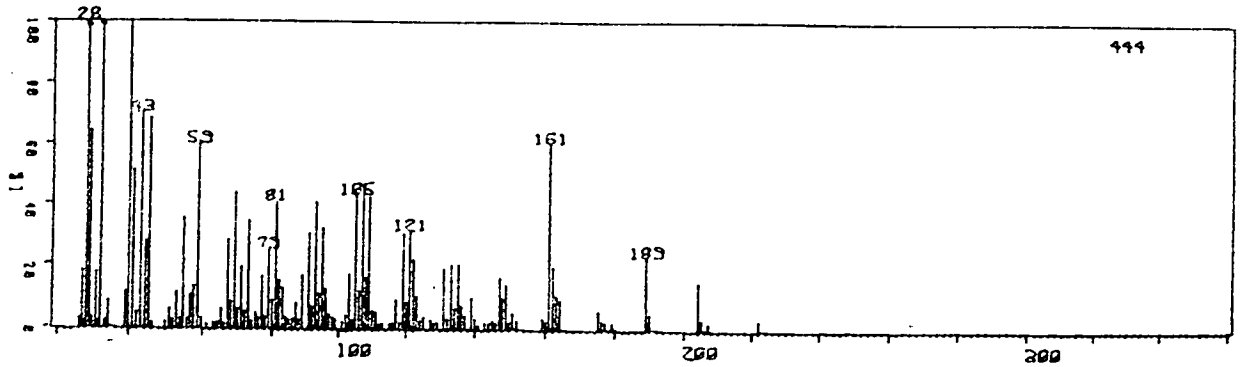
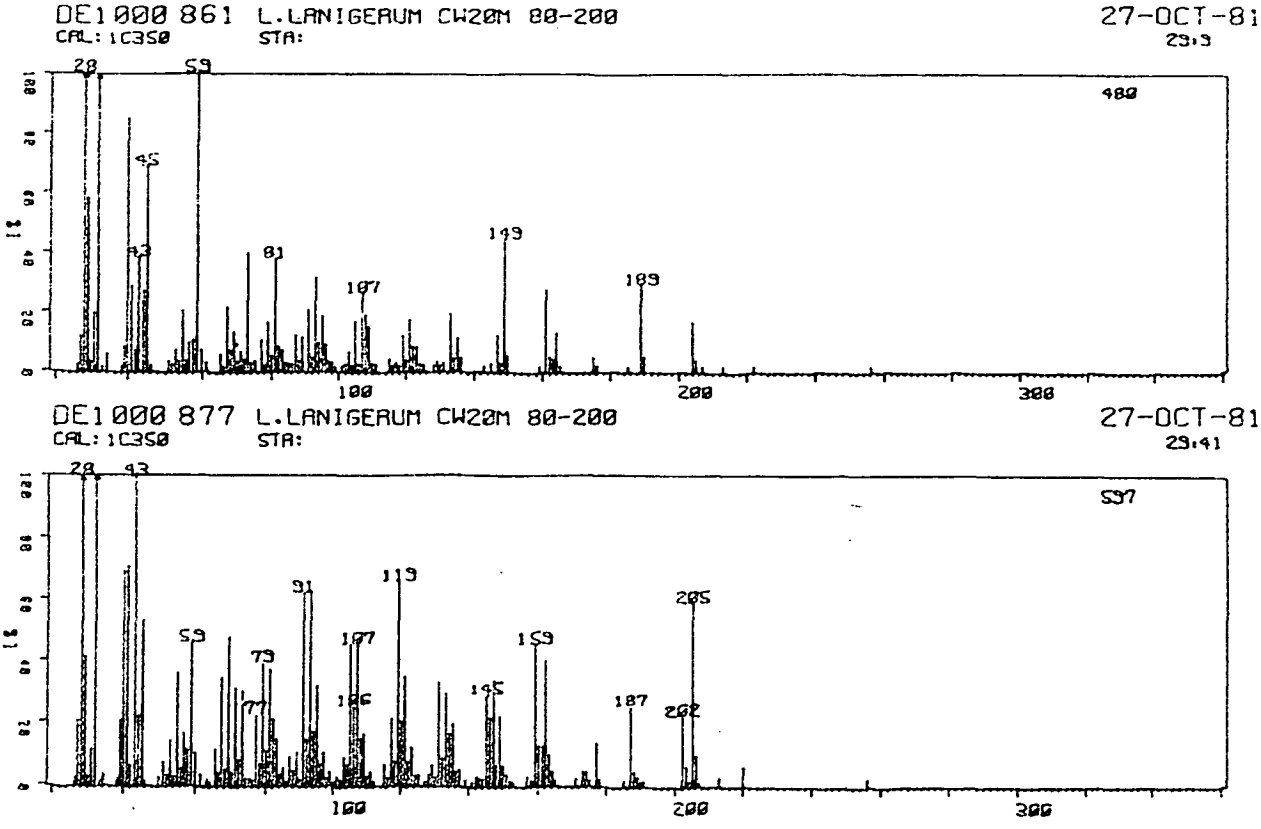
CAL: 1C3S0 STA: 28:38

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CAL: 1C3S0
STA:
27-OCT-81
29:9
480
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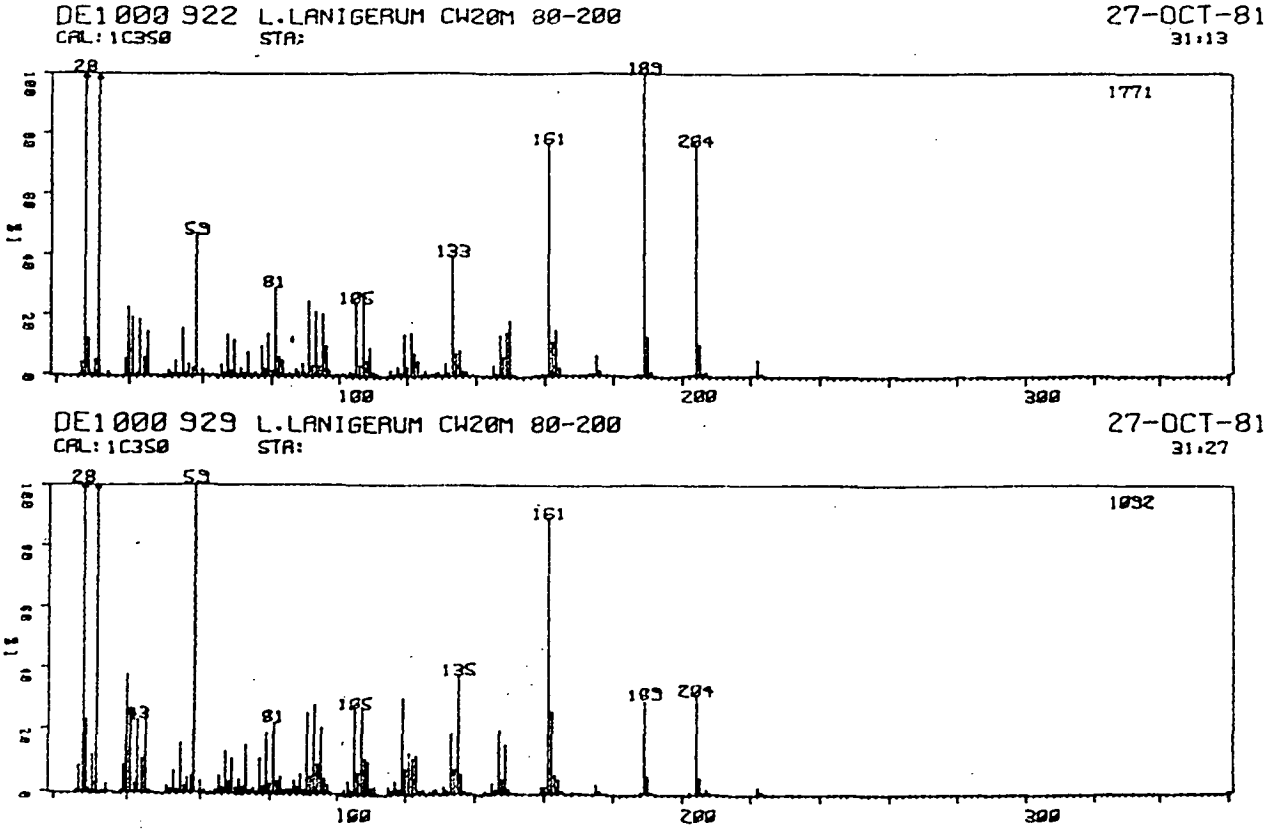
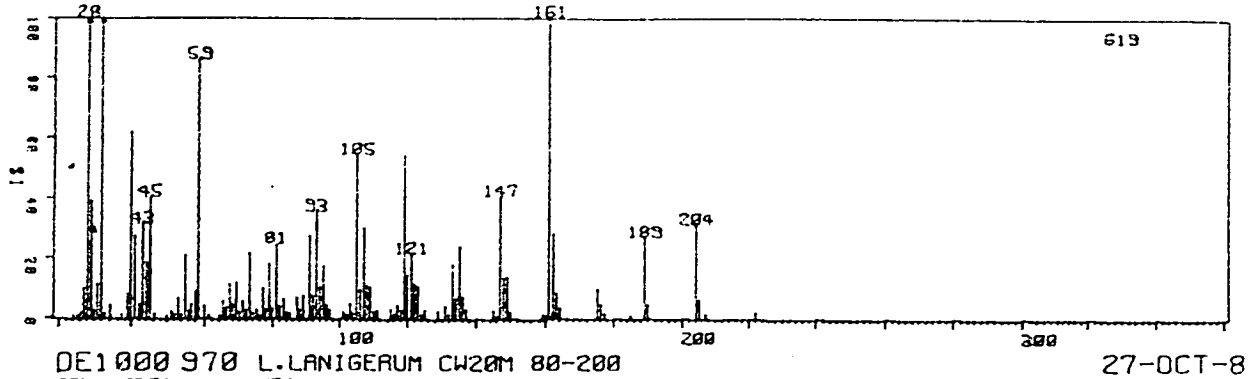
CAL: 1C3S0
STA:
27-OCT-81
29:41
537


DE1000 922 L.LANIGERUM CW20M 80-200
CAL: 1C3S0
STA:
27-OCT-81
31:13
1771
DE1000 929 L.LANIGERUM CW20M 80-200
CAL: 1C3S0
STA:
27-OCT-81
31:27
1092

DE1000 937 L.LANIGERUM CW20M 80-200 27-OCT-81
CAL: 1C3S0 STA: 31:43



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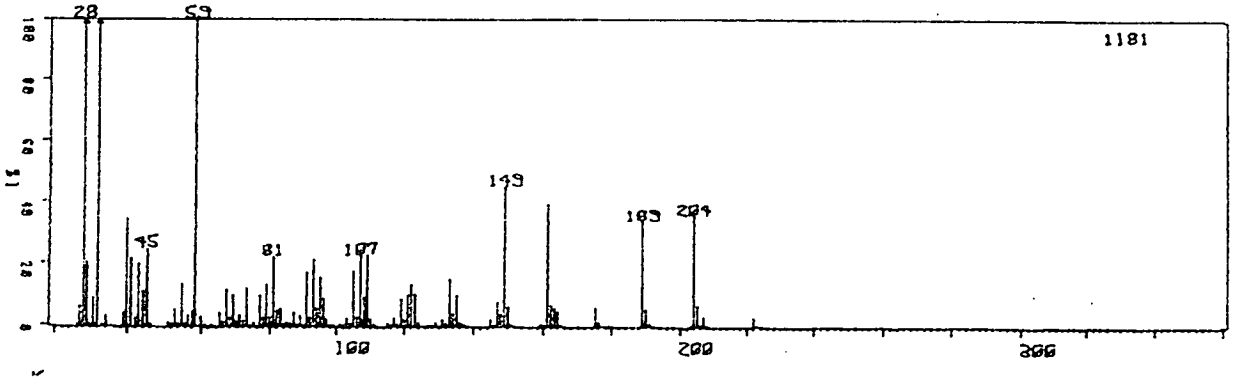

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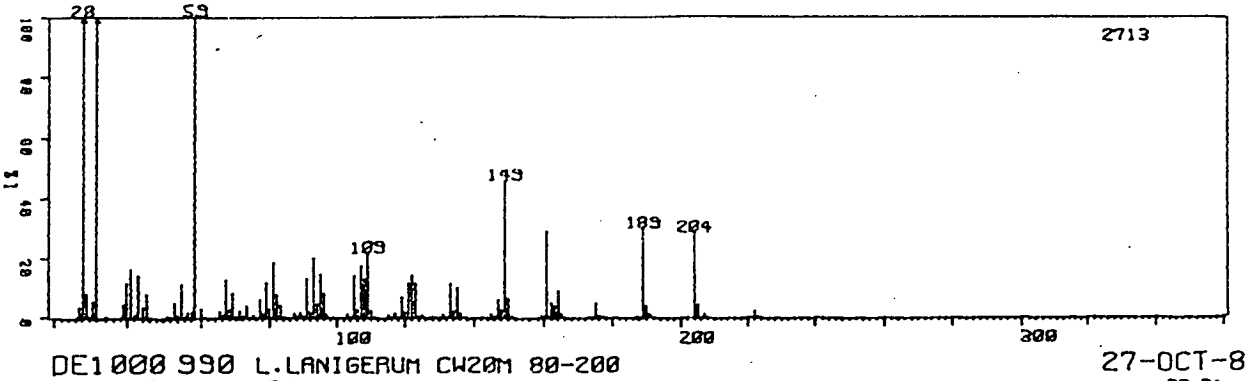

DE1000 990 L.LANIGERUM CW20M 80-200 27-OCT-81
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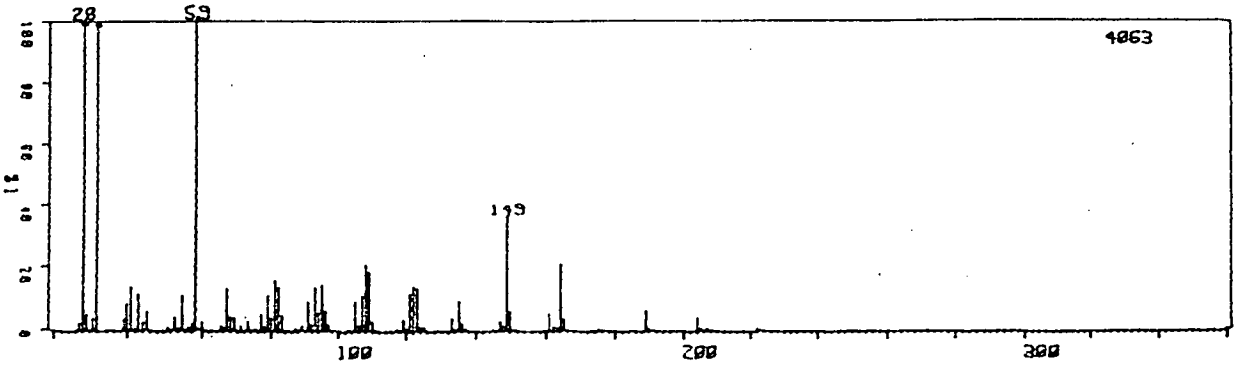
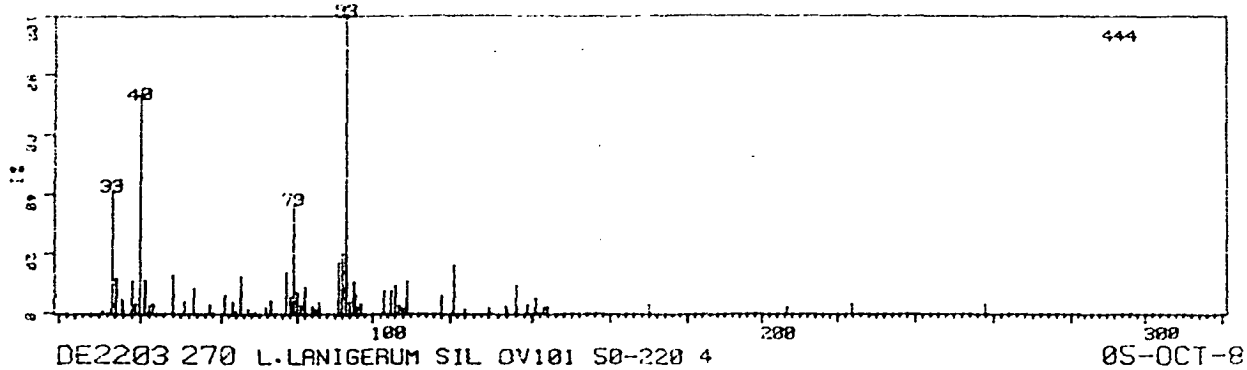


APPENDIX 5

Mass spectra of components of L. lanigerum separated on OV-101 phase with reference to total ion current trace Fig. 12.

DE2203 232 L.LANIGERUM SIL OV101 S0-220 4 05-OCT-82
CAL:1DSS0 STA:E. 7:40



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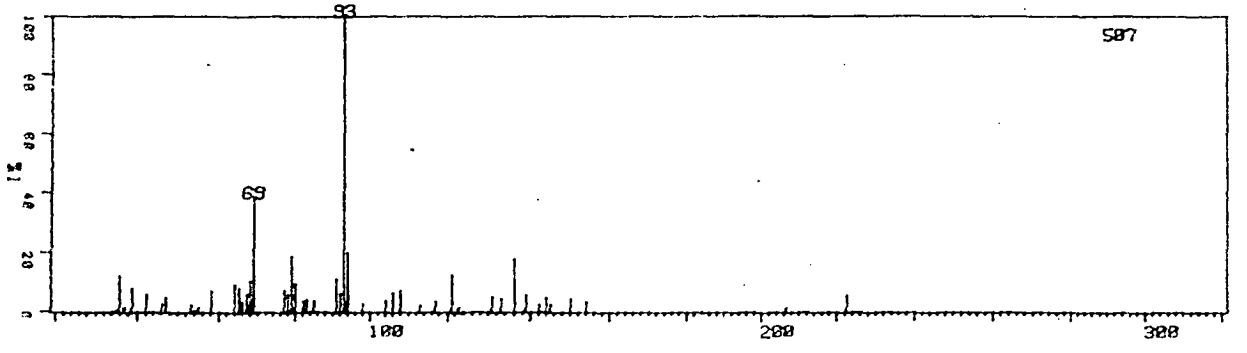
CAL:1DSS0 STA:E. 8:55



DE2203 322 L.LANIGERUM SIL OV101 S0-220 4 05-OCT-82
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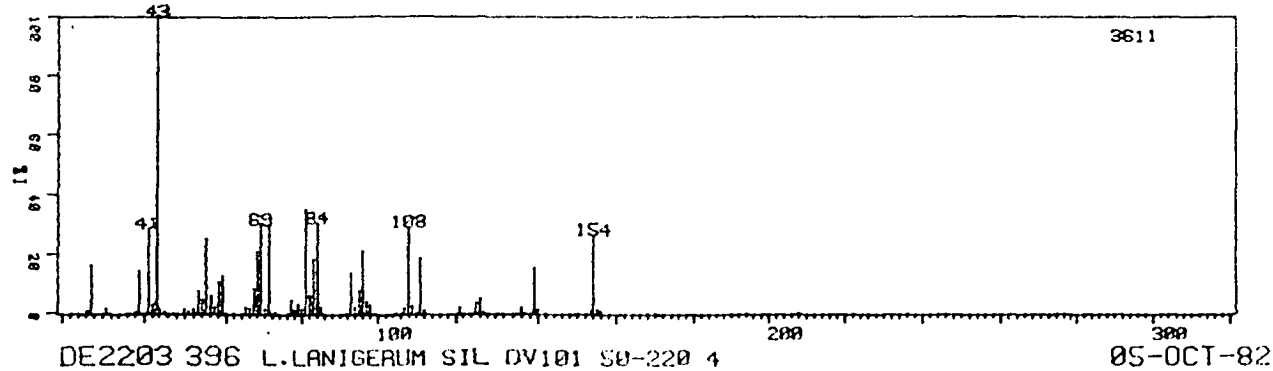

DE2203 396 L.LANIGERUM SIL OV101 S0-220 4 05-OCT-82
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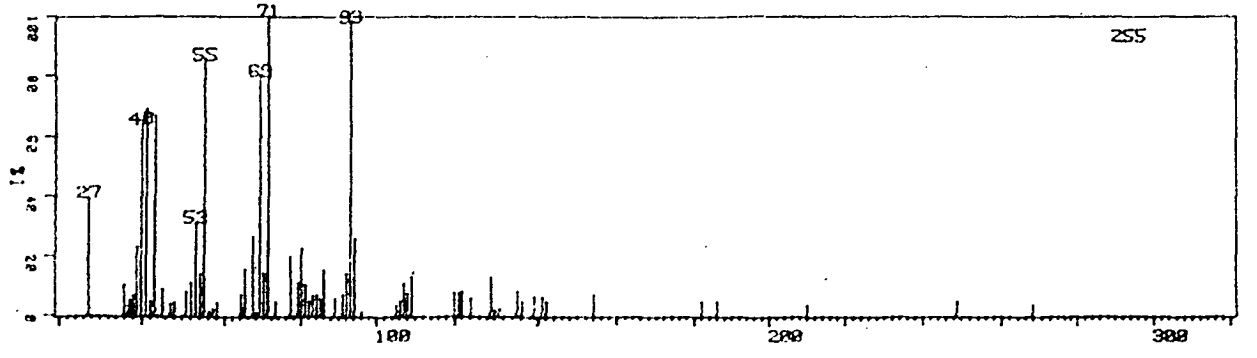
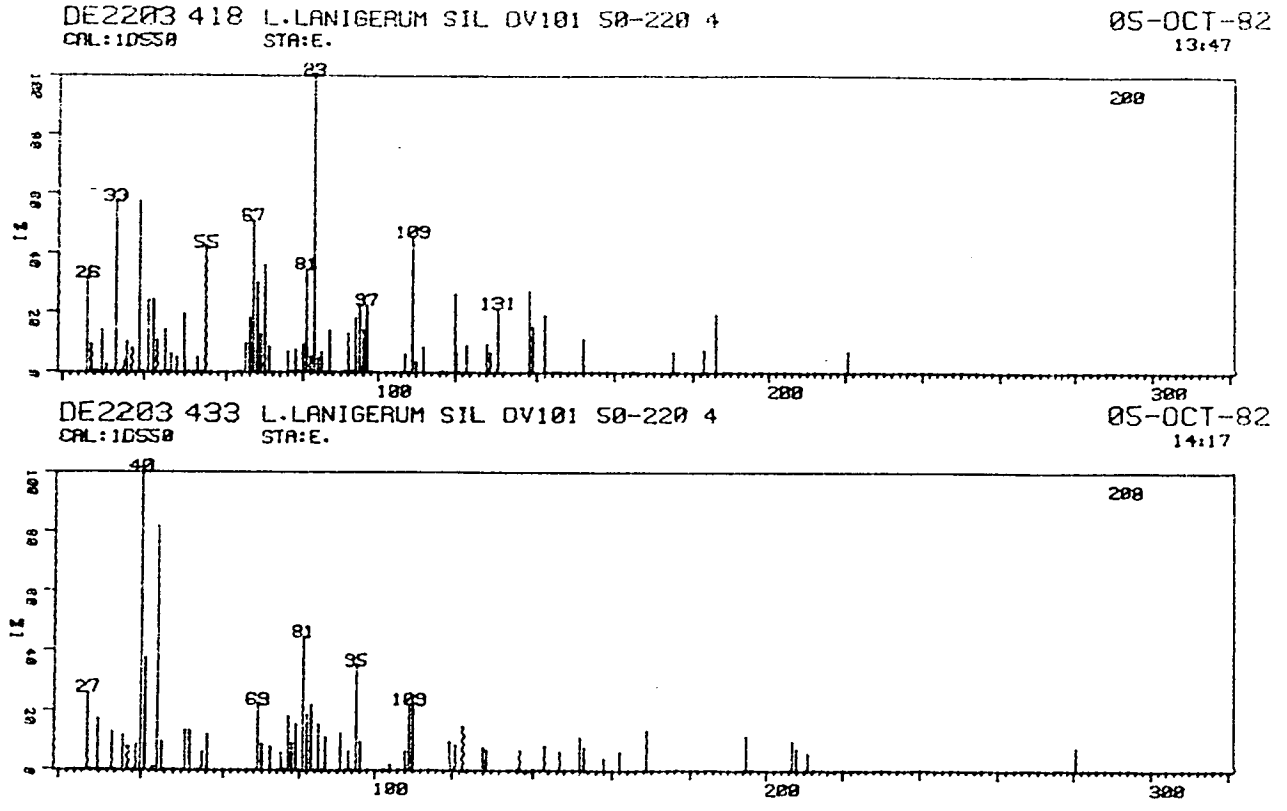
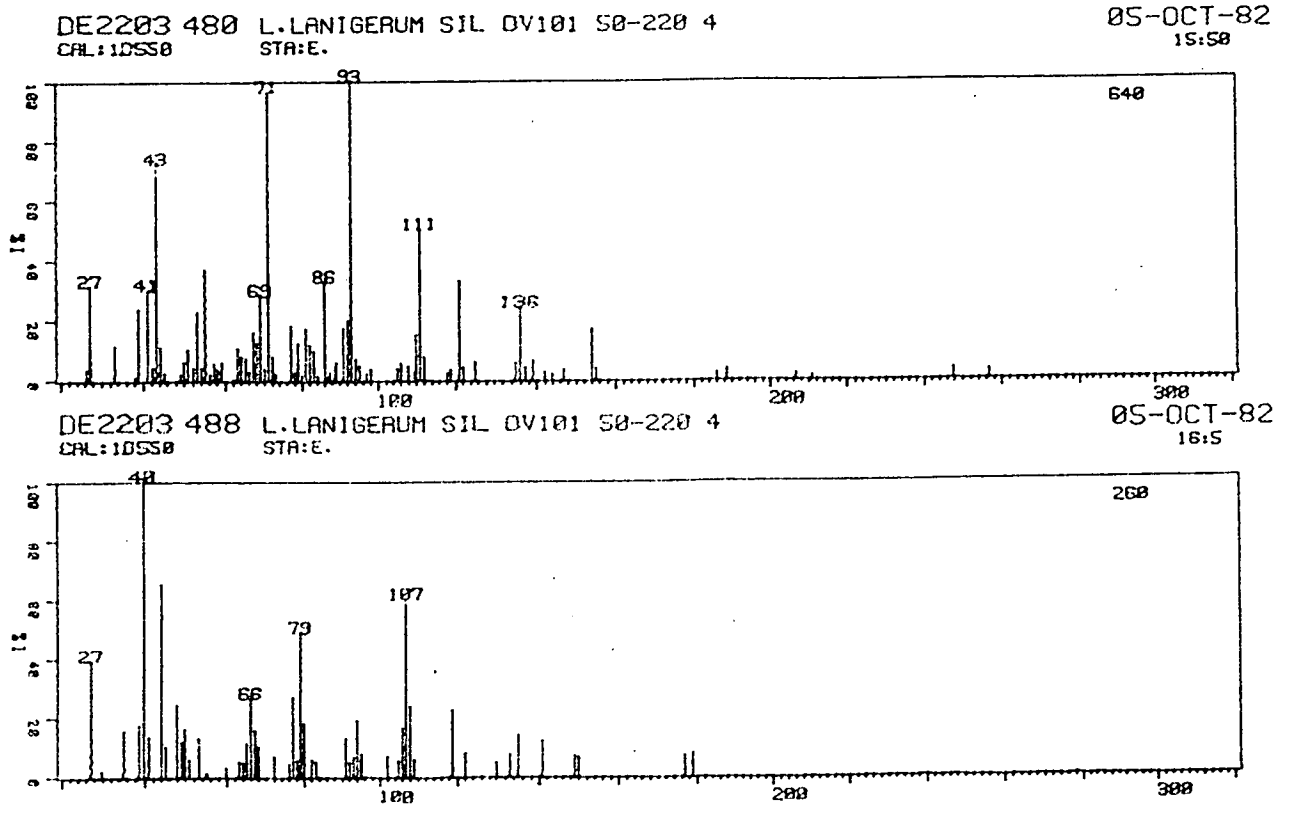
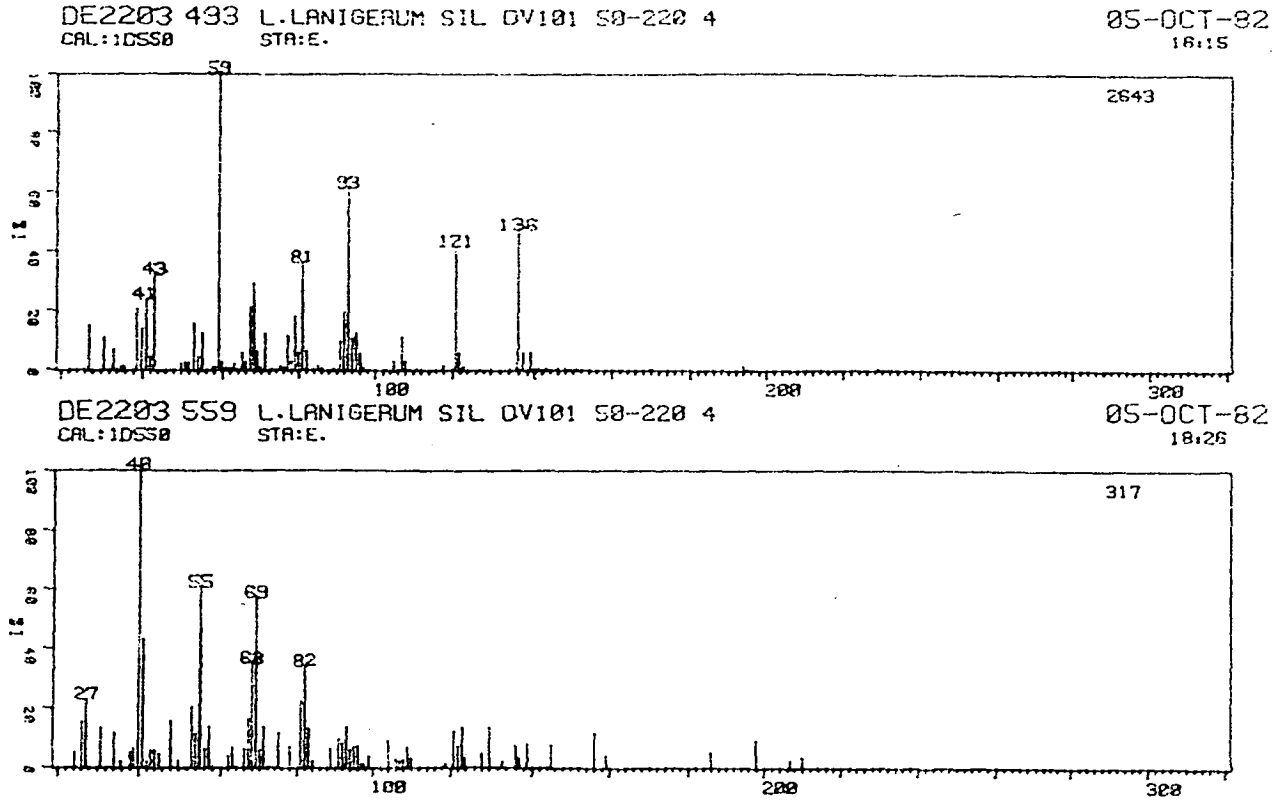
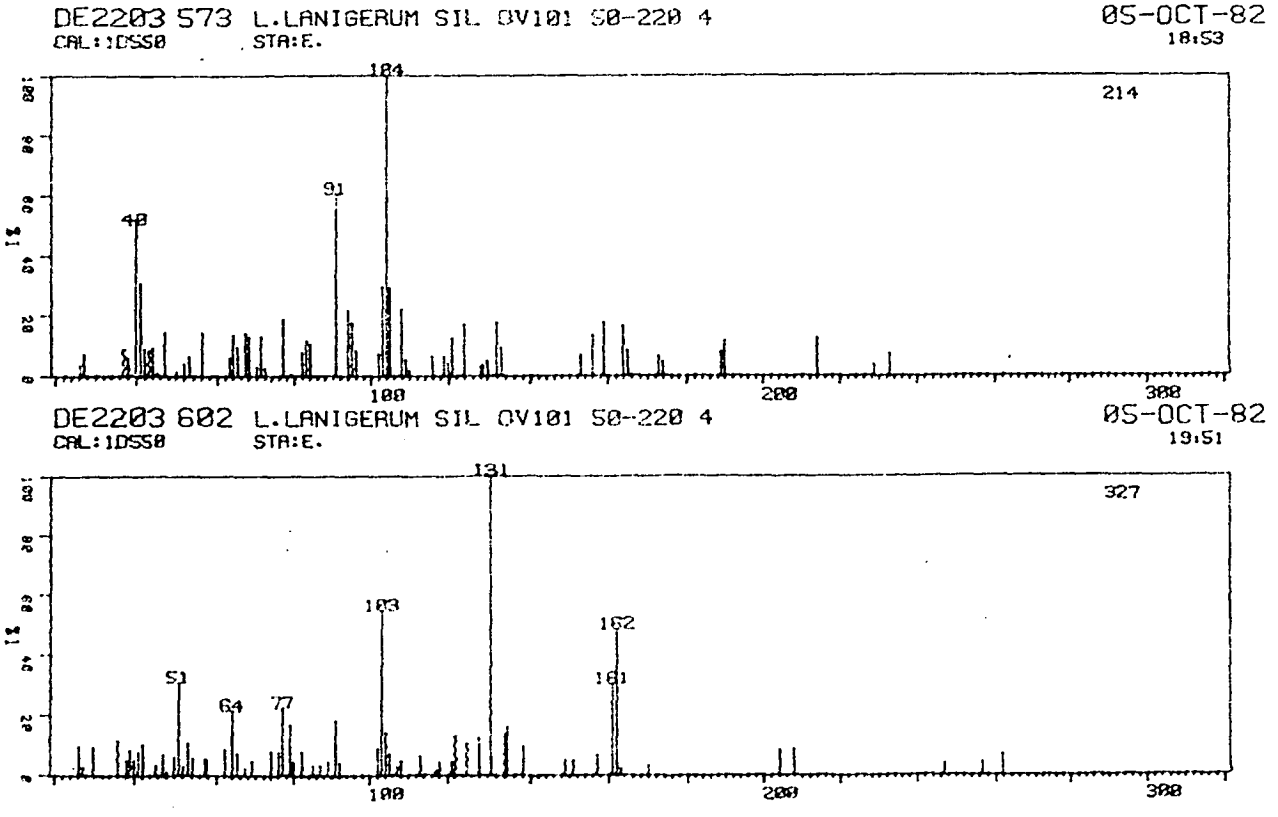


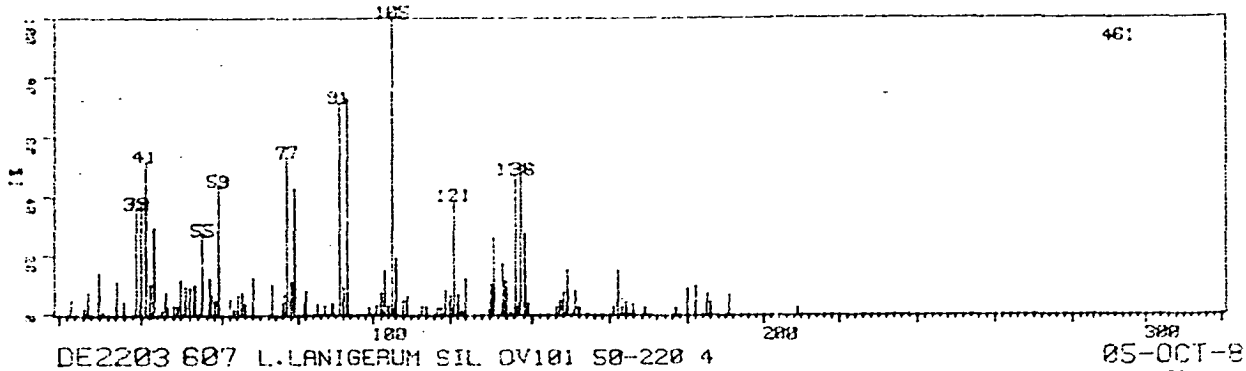

DE2203 418 L.LANIGERUM SIL OV101 50-220 4
CAL:1DSS0 STA:E.
05-OCT-82
13:47
200
23
39
57
55
81
109
26
37
131
100
200
300
DE2203 433 L.LANIGERUM SIL OV101 50-220 4
CAL:1DSS0 STA:E.
05-OCT-82
14:17
200
40
81
95
27
69
109
100
200
300


DE2203 480 L.LANIGERUM SIL OV101 50-220 4
CAL:1DSS0 STA:E.
05-OCT-82
15:50
640
93
71
43
111
27
41
69
86
136
100
200
300
DE2203 488 L.LANIGERUM SIL OV101 50-220 4
CAL:1DSS0 STA:E.
05-OCT-82
16:5
260
40
107
79
27
66
100
200
300


DE2203 433 L.LANIGERUM SIL OV101 50-220 4
CAL:1DSS0 STA:E.
05-OCT-82
16:15
2643
59
93
136
121
81
43
41
100
200
300
DE2203 559 L.LANIGERUM SIL OV101 50-220 4
CAL:1DSS0 STA:E.
05-OCT-82
18:26
317
40
55
69
68
82
27
100
200
300


DE2203 573 L.LANIGERUM SIL OV101 50-220 4
CAL:1DSS0 STA:E.
05-OCT-82
18:53
214
104
91
40
100
200
300
DE2203 602 L.LANIGERUM SIL OV101 50-220 4
CAL:1DSS0 STA:E.
05-OCT-82
19:51
327
131
103
162
161
51
64
77
100
200
300

DE2203 604 L.LANIGERUM SIL OV101 50-220 4 05-OCT-82
CAL:1DSS0 STA:E. 19:55



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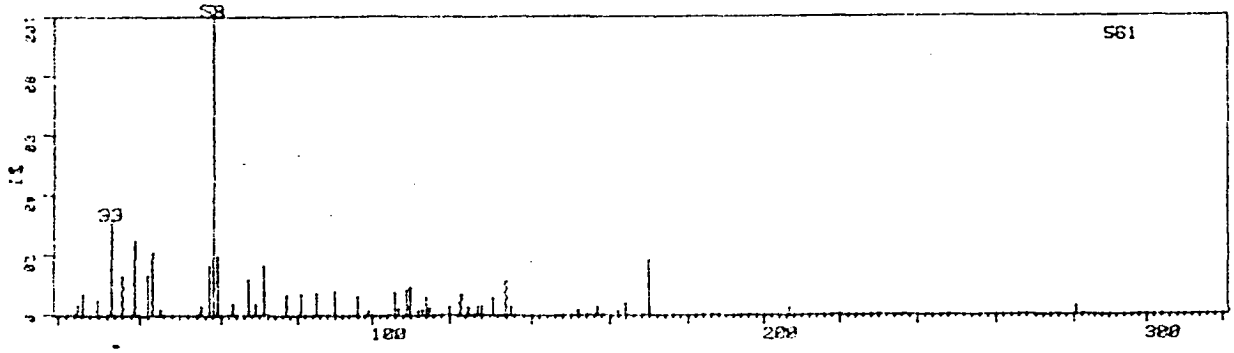

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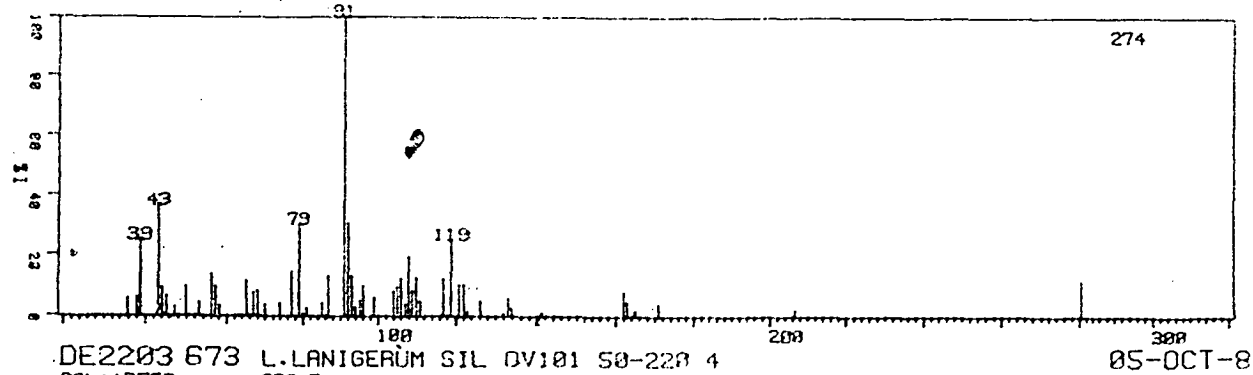

DE2203 673 L.LANIGERUM SIL OV101 50-220 4 05-OCT-82
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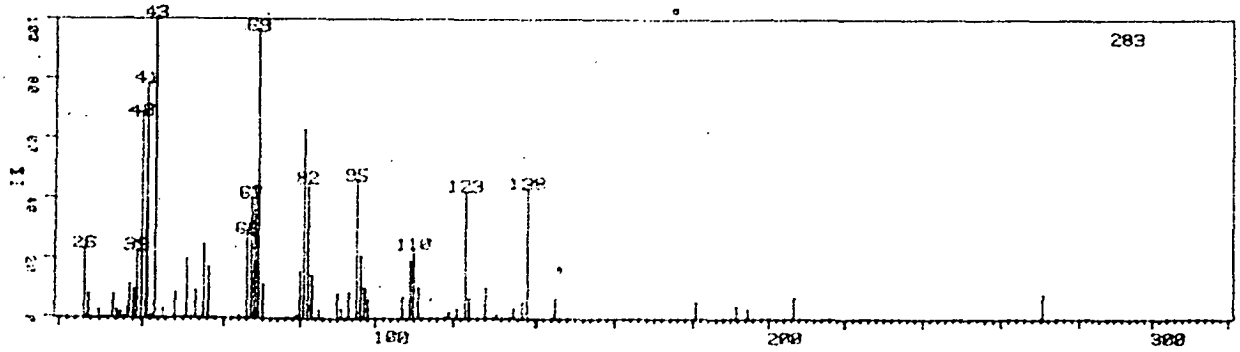

DE2203 685 L.LANIGERUM SIL OV101 50-220 4 05-OCT-82
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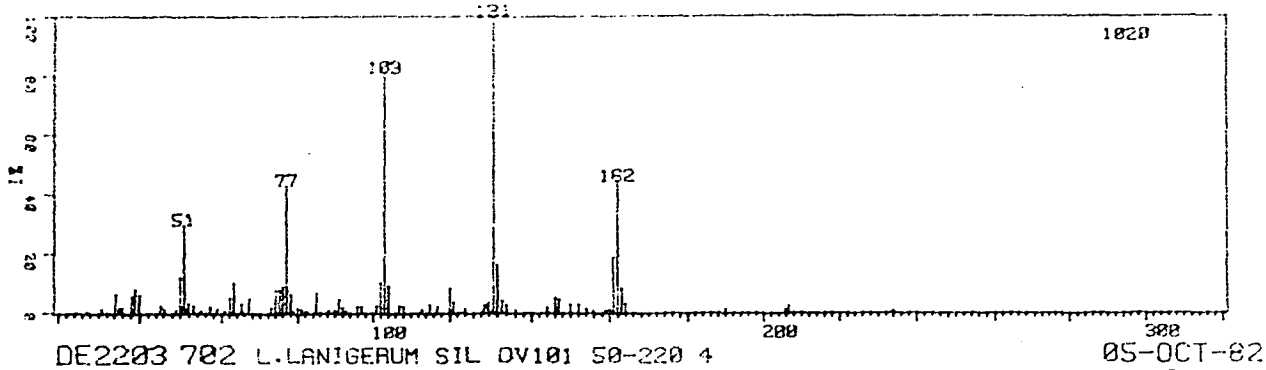

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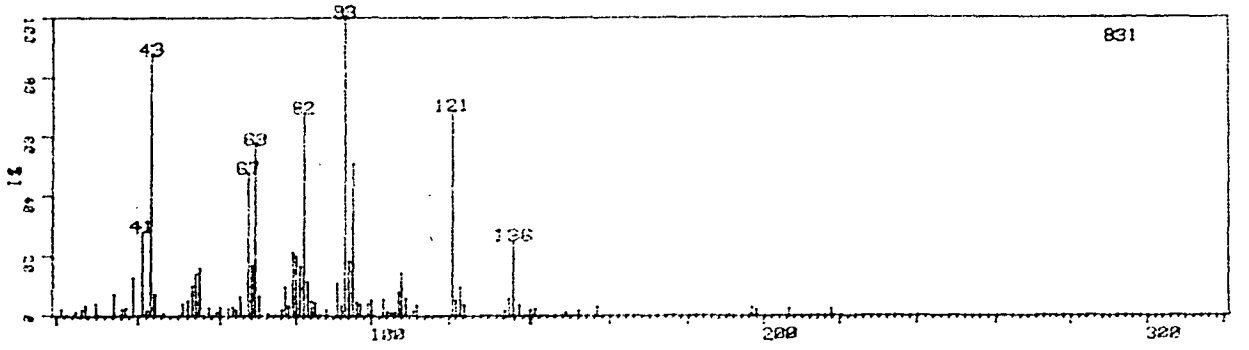

DE2203 709 L.LANIGERUM SIL OV101 50-220 4 05-OCT-82
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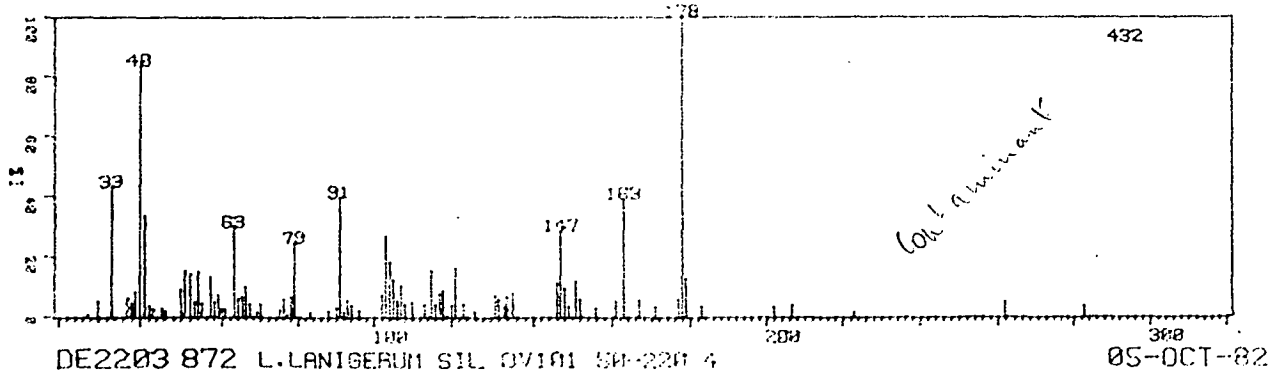

DE2203 872 L.LANIGERUM SIL OV101 50-220 4 05-OCT-82
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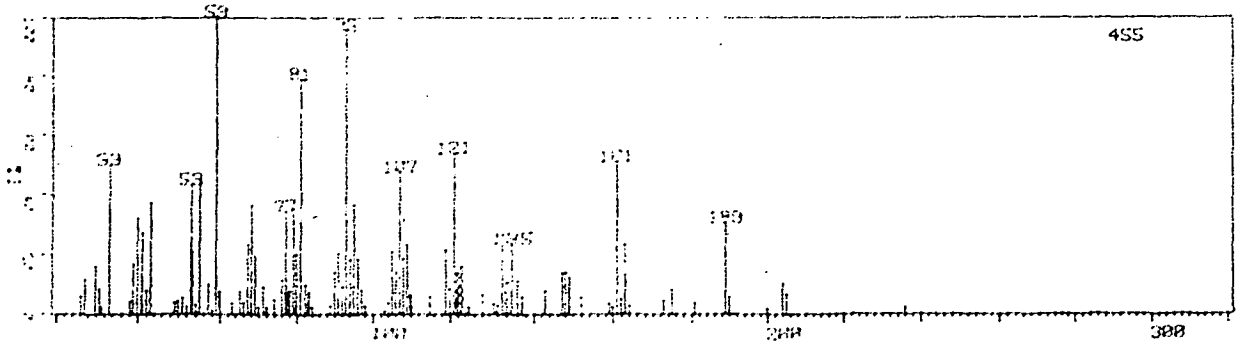

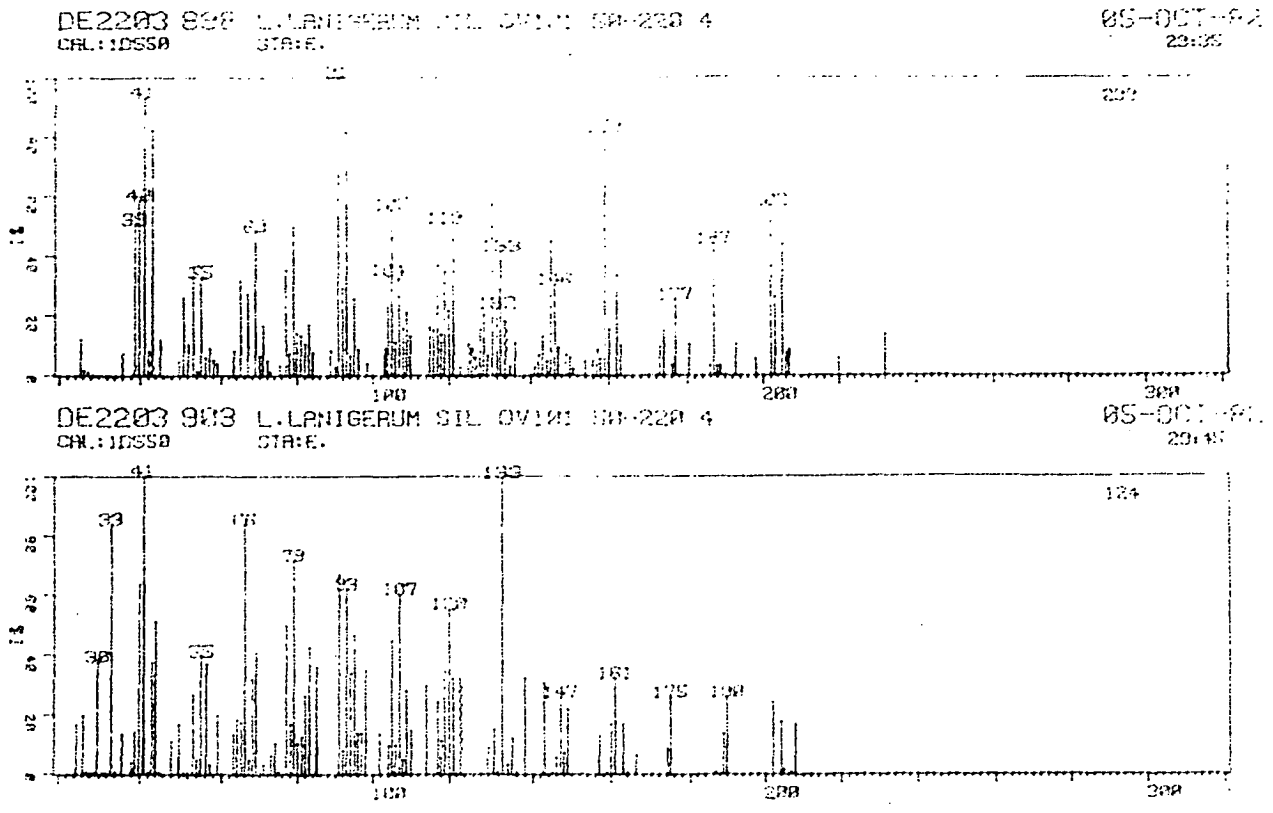
DE2203 898
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05-OCT-82
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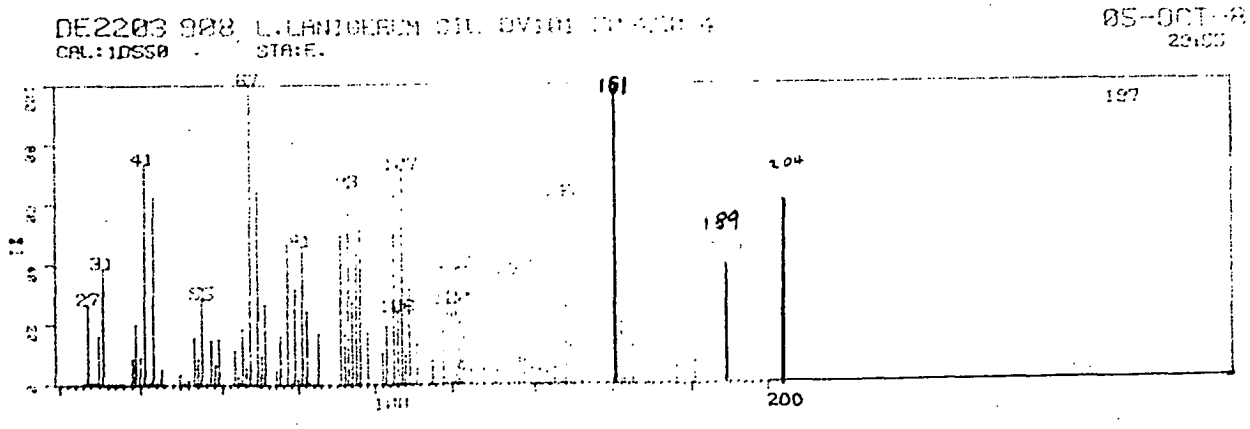
CAL:1DSS0
STA:E.
05-OCT-82


DE2203 908
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STA:E.
05-OCT-82
161
197
189
204
200

DE2203 915 L.LANIGERUM SIL OV101 50-220 4
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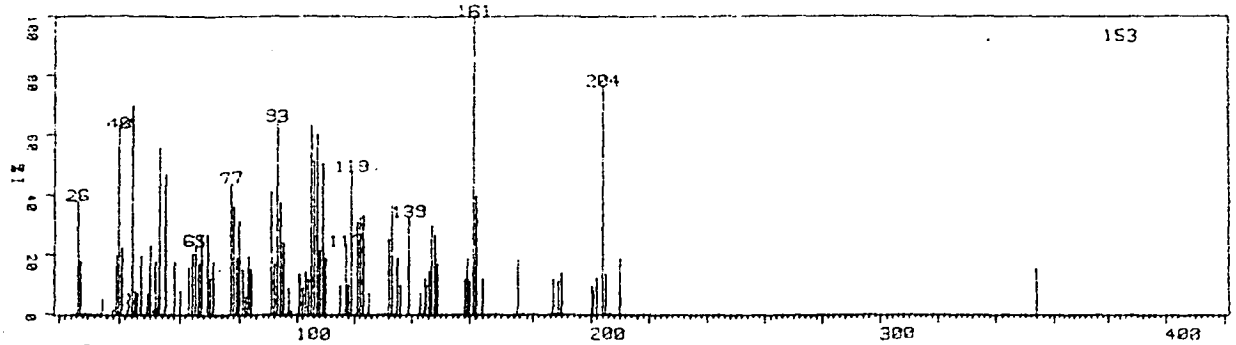

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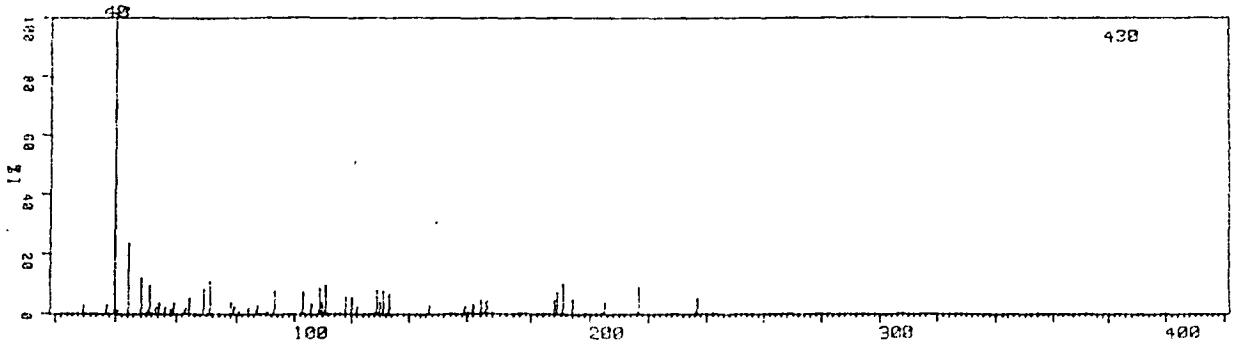

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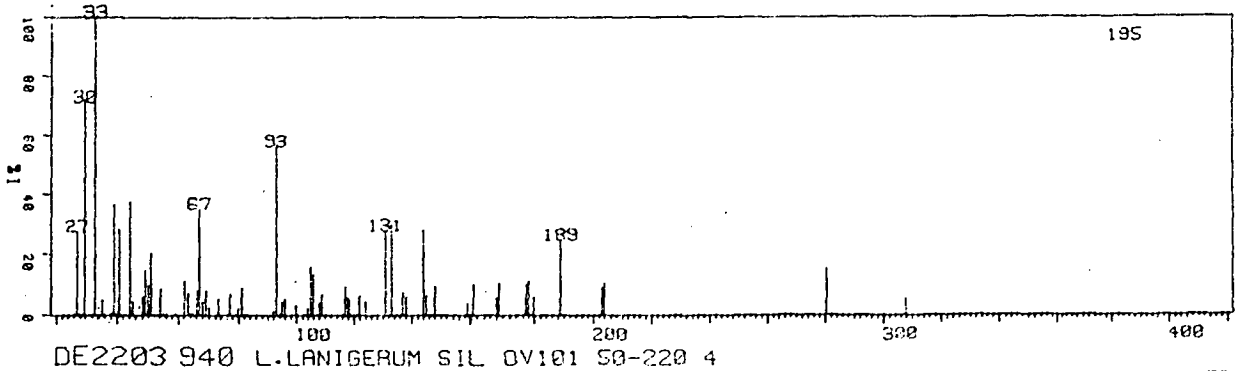

DE2203 940 L.LANIGERUM SIL OV101 50-220 4
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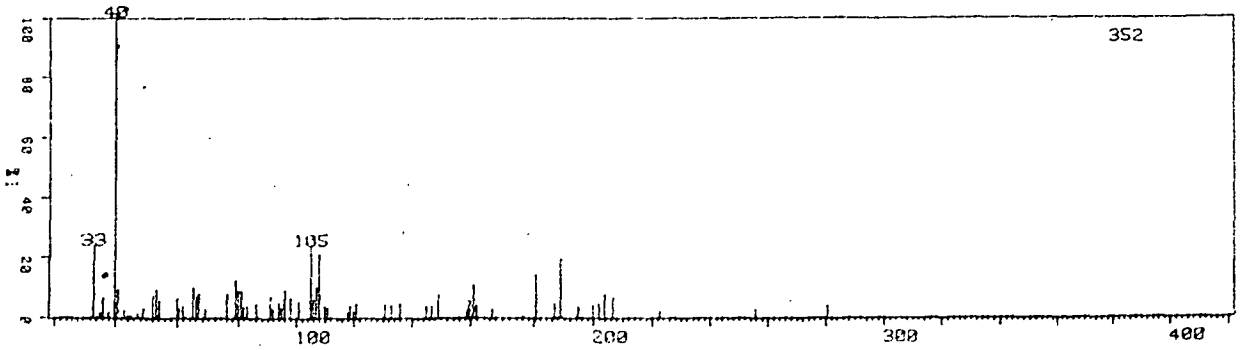

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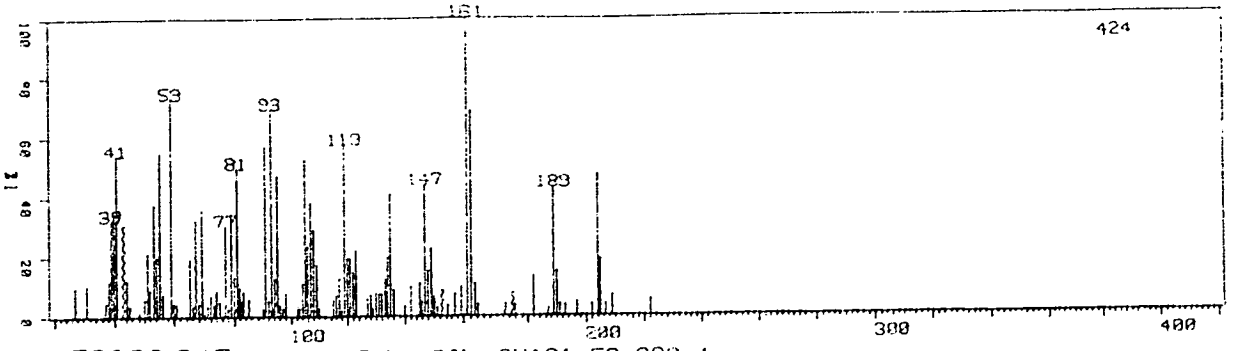
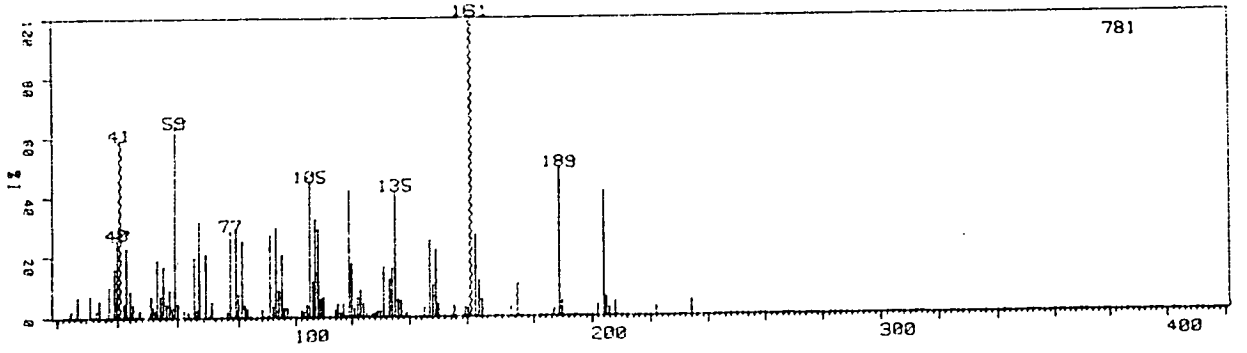


DE2203 947 L.LANIGERUM SIL OV101 50-220 4
CAL:1DS50 STA:E. 31:12



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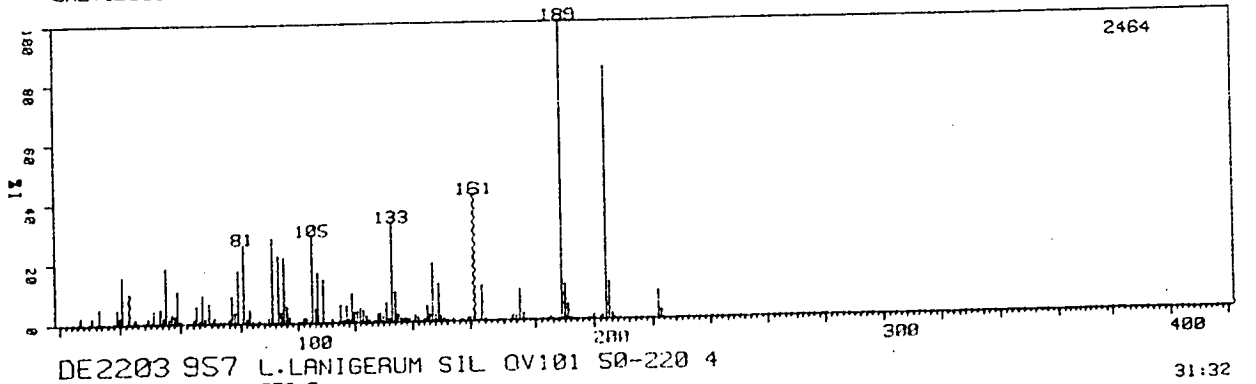
CAL:1DS50 STA:E. 31:20



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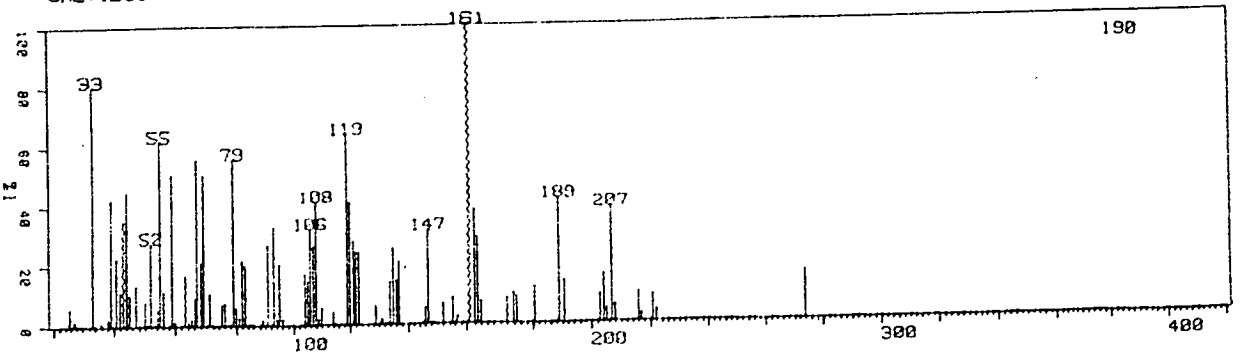
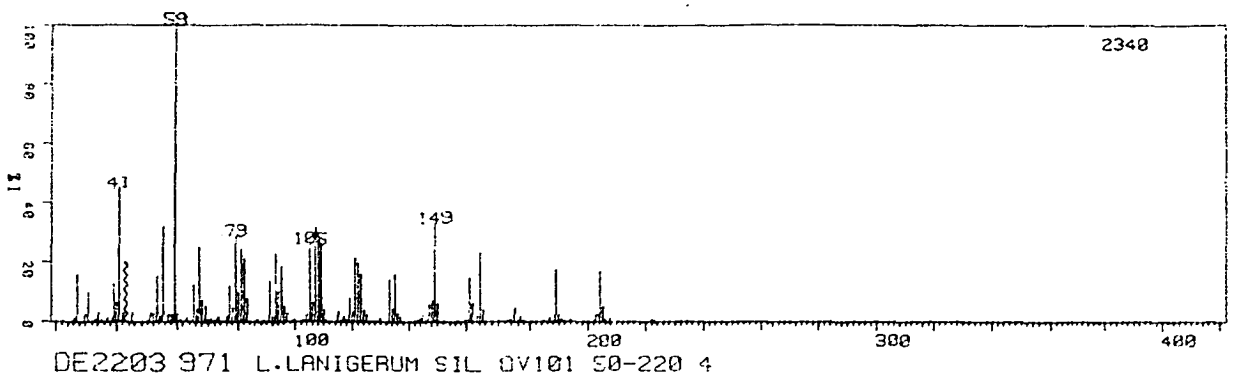
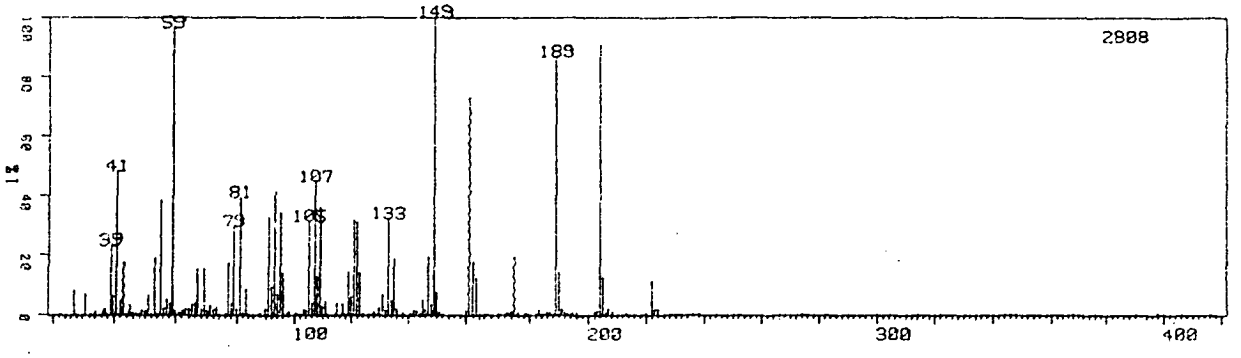


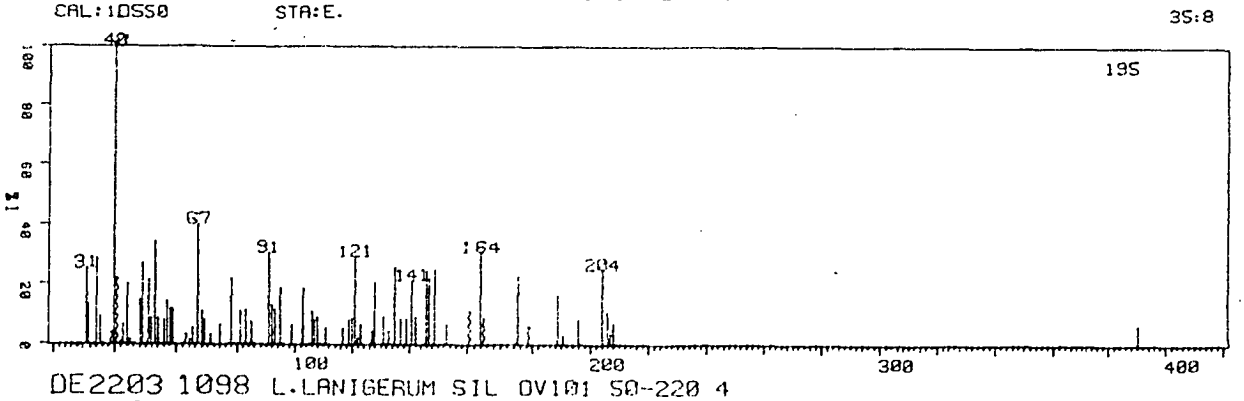
DE2203 964 L.LANIGERUM SIL OV101 50-220 4
CAL:1DSS0 STA:E. 31:46



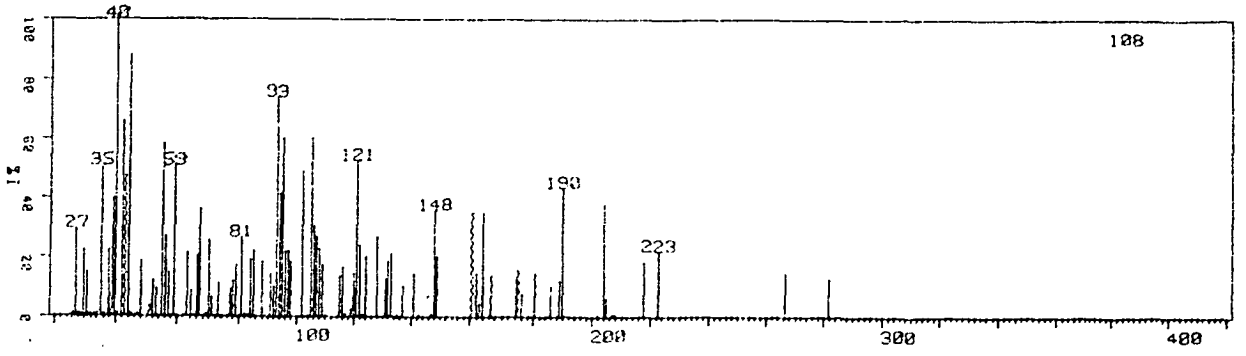
DE2203 971 L.LANIGERUM SIL OV101 50-220 4
CAL:1DSS0 STA:E. 32:0

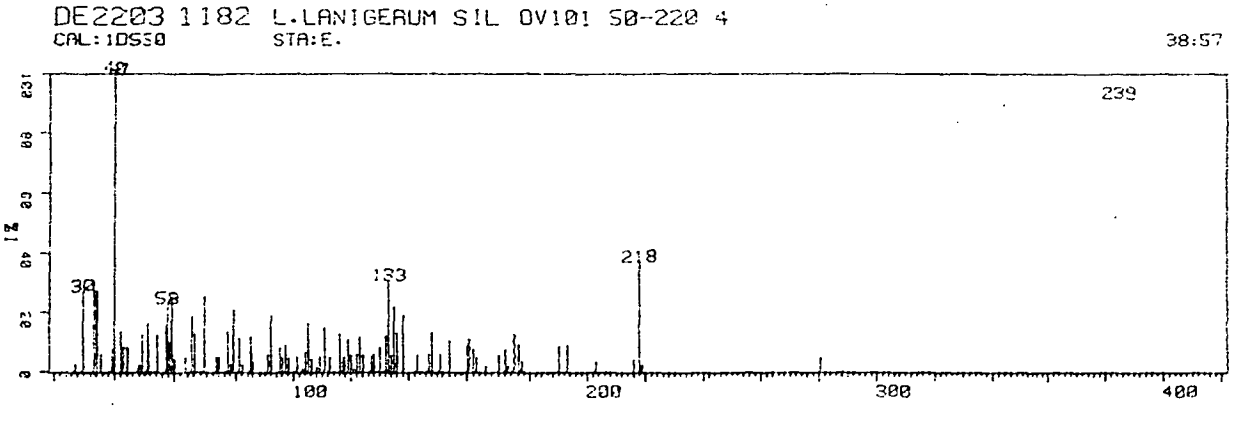


DE2203 1066 L.LANIGERUM SIL OV101 50-220 4
CAL:1DSS0 STA:E. 35:8



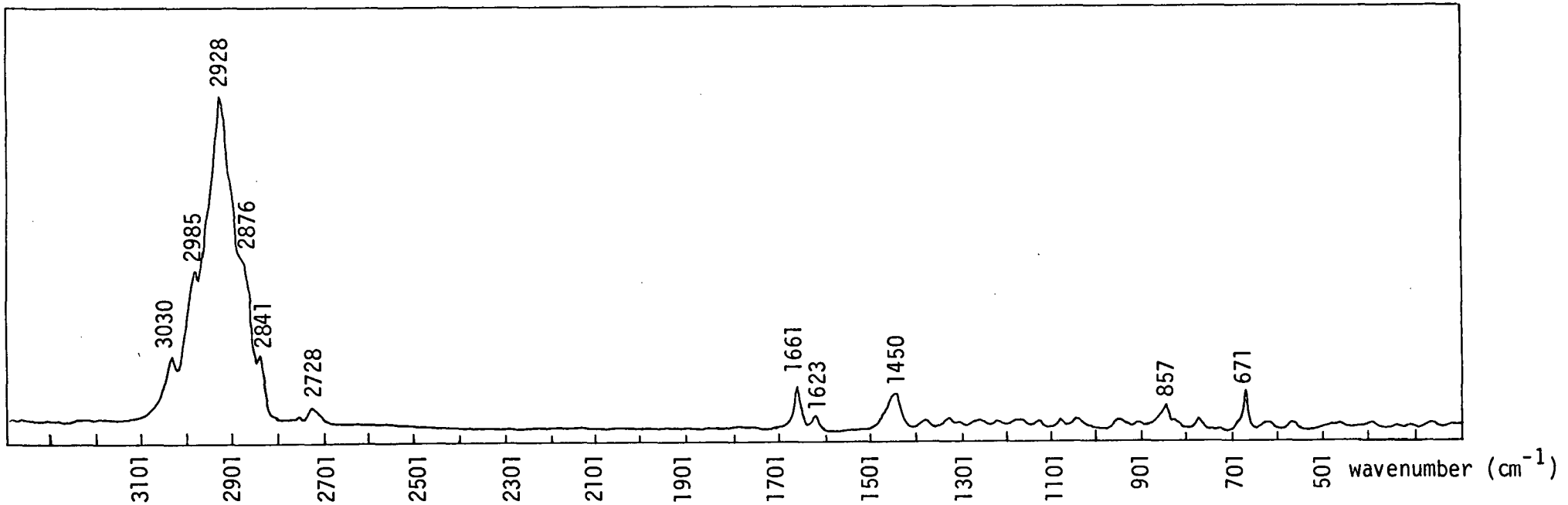
DE2203 1098 L.LANIGERUM SIL OV101 50-220 4
CAL:1DSS0 STA:E. 36:11




DE2203 1182 L.LANIGERUM SIL OV101 S0-220 4
CAL:1DS50
STA:E.
38:57
239
218
133
58
30
100
200
300
400

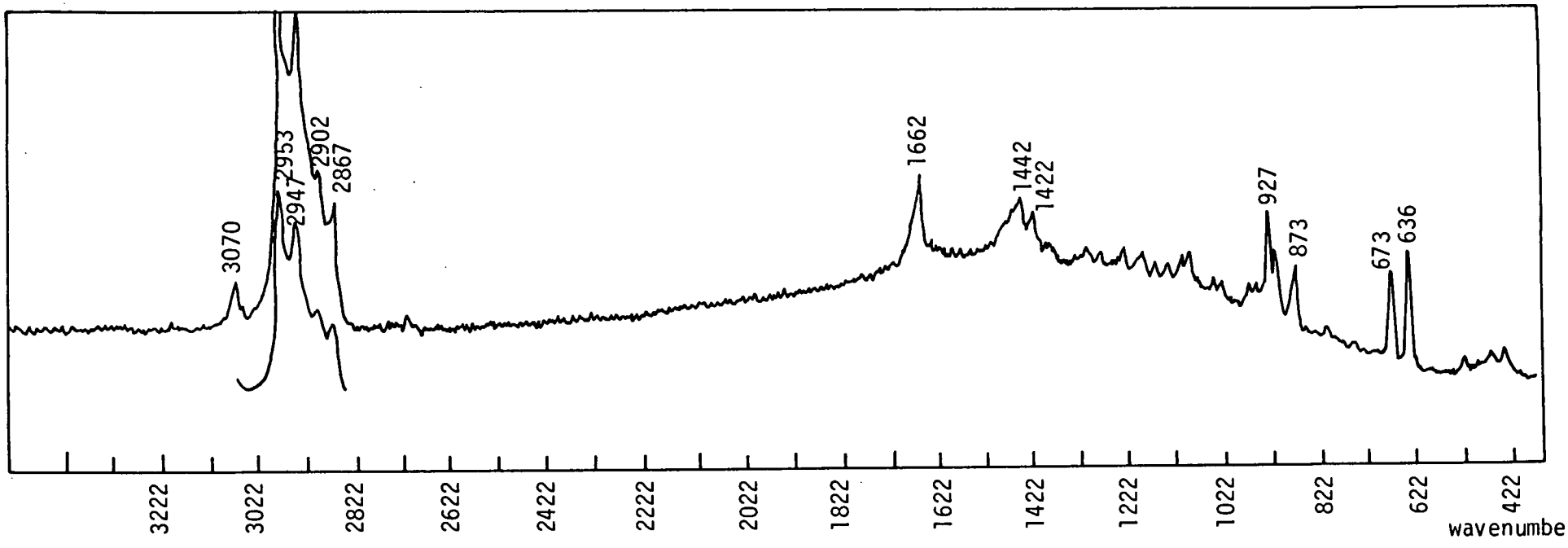
APPENDIX 6

Raman spectra of components isolated by GC from Atherosperma moschatum.



Laser Raman Spectra of Trapped Component No. 2 from A. moschatum.
Identified as α-pinene.

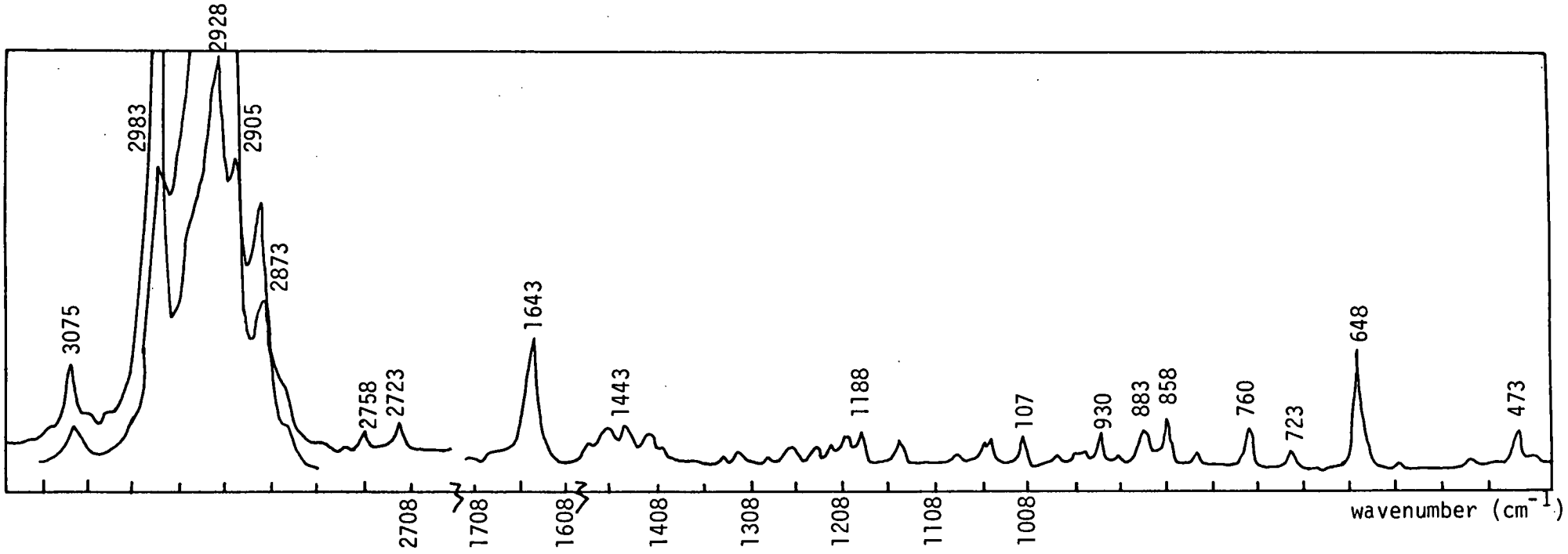
Sens. 4000 c/s
SBW 8.0 cm^{-1}
PP 10 secs.



Laser Raman Spectrum of Trapped Component No. 3 from Atherosperma moschatum.

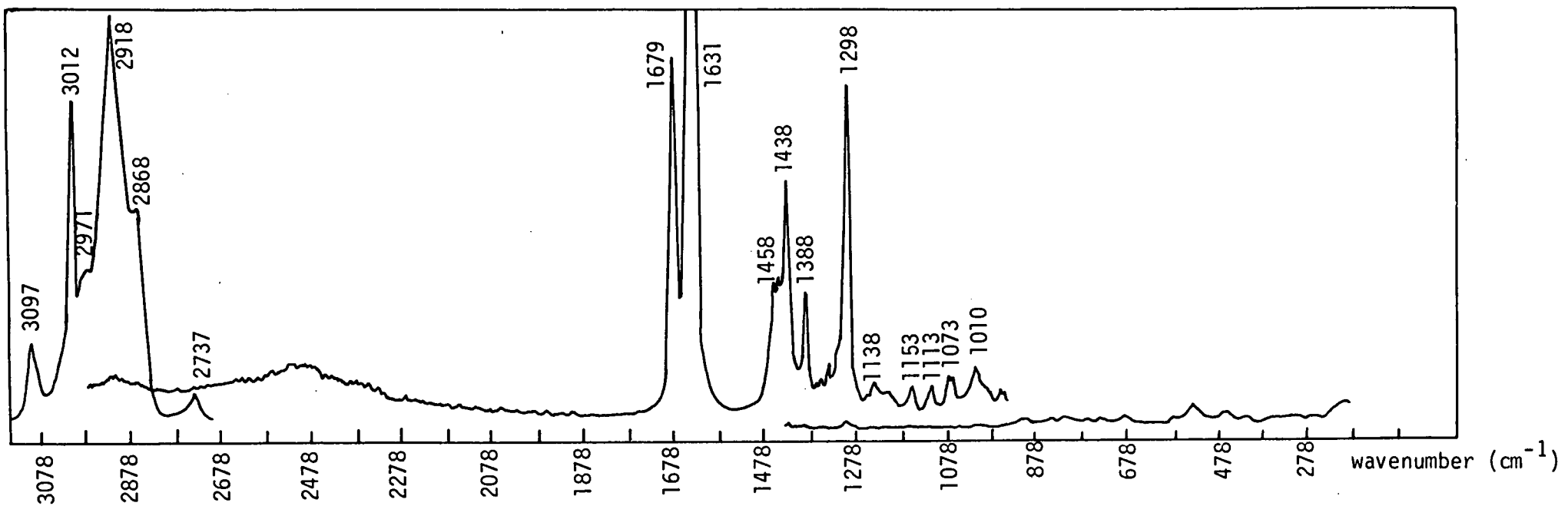
Identified as Camphene.

Sens. 2500 c/s
SBW 8.0 cm^{-1}
PP 5 secs.



Laser Raman Spectra of Trapped Component No. 4 from Atherosperma moschatum.
Identified as β-pinene.

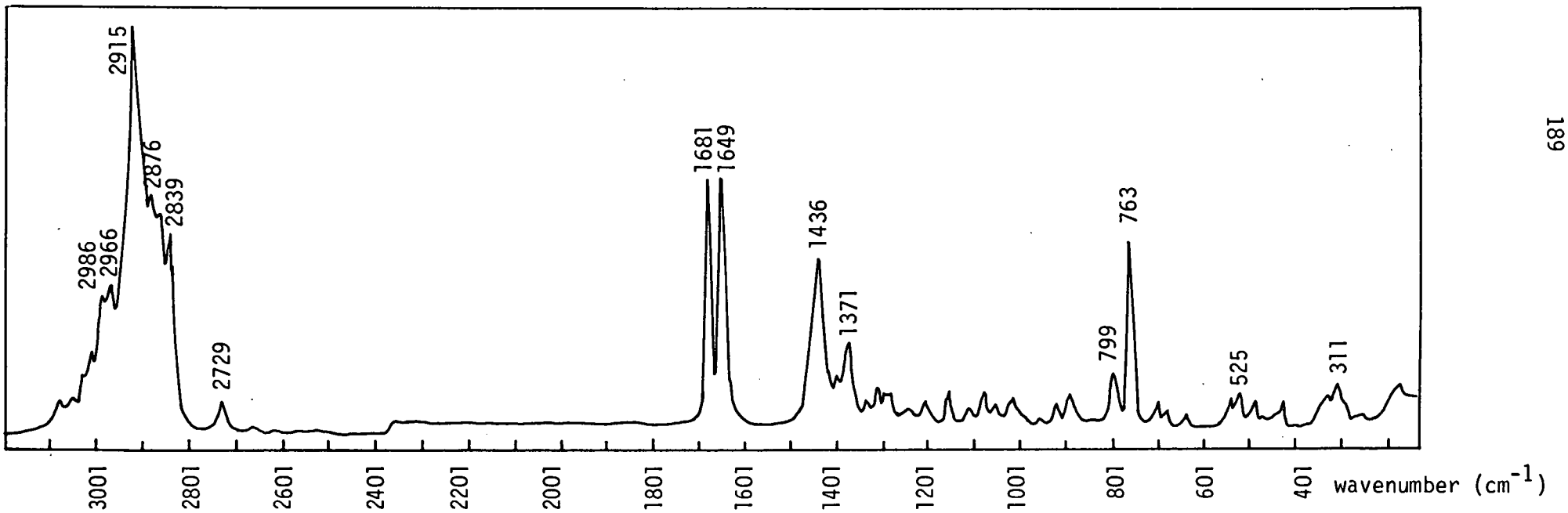
Sens. 2000 c/s
SBW 10 cm^{-1}
PP 10 secs.



Laser Raman Spectrum of Trapped Component No. 5 from Atherosperma moschatum.

Identified as Myrcene.

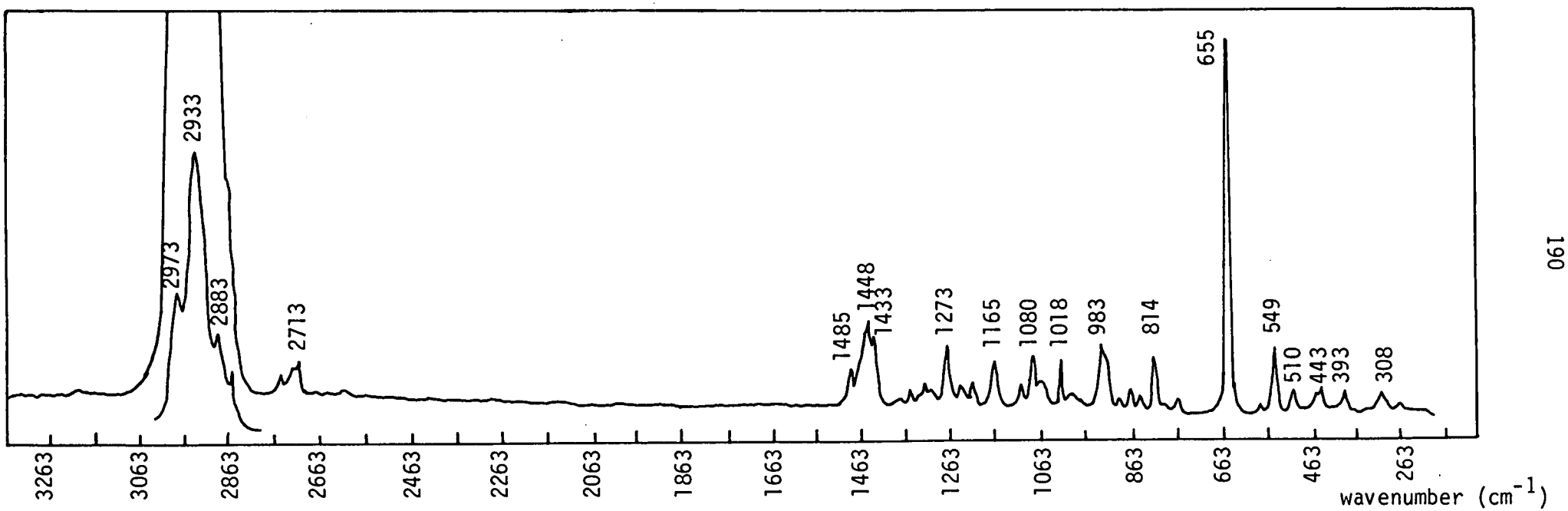
Sens. 200 c/s
SBW 3.0 cm^{-1}
PP 10 secs.



Laser Raman Spectrum of Trapped Component No. 6 from Atherosperma moschatum.

Identified as -Limonene.

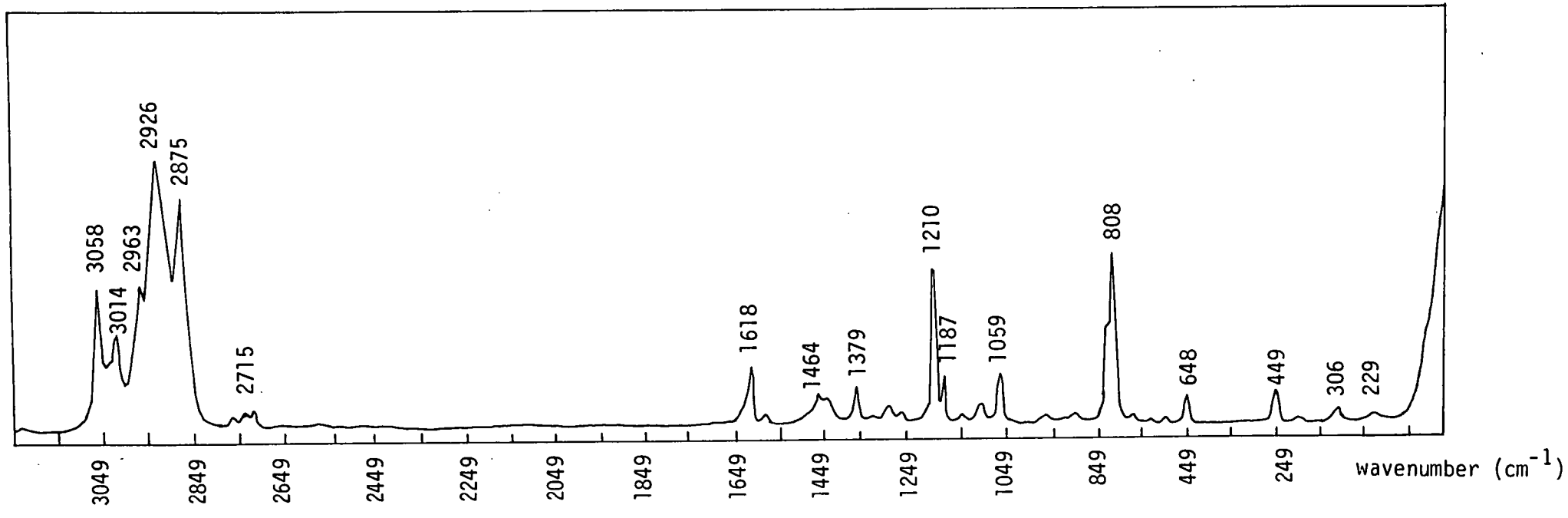
Sens. 2000 c/s
SBW 8.0 cm^{-1}
PP 10 secs.



Laser Raman Spectrum of Trapped Component No. 7 from Atherospera moschatum.

Identified as 1.8 Cineole.

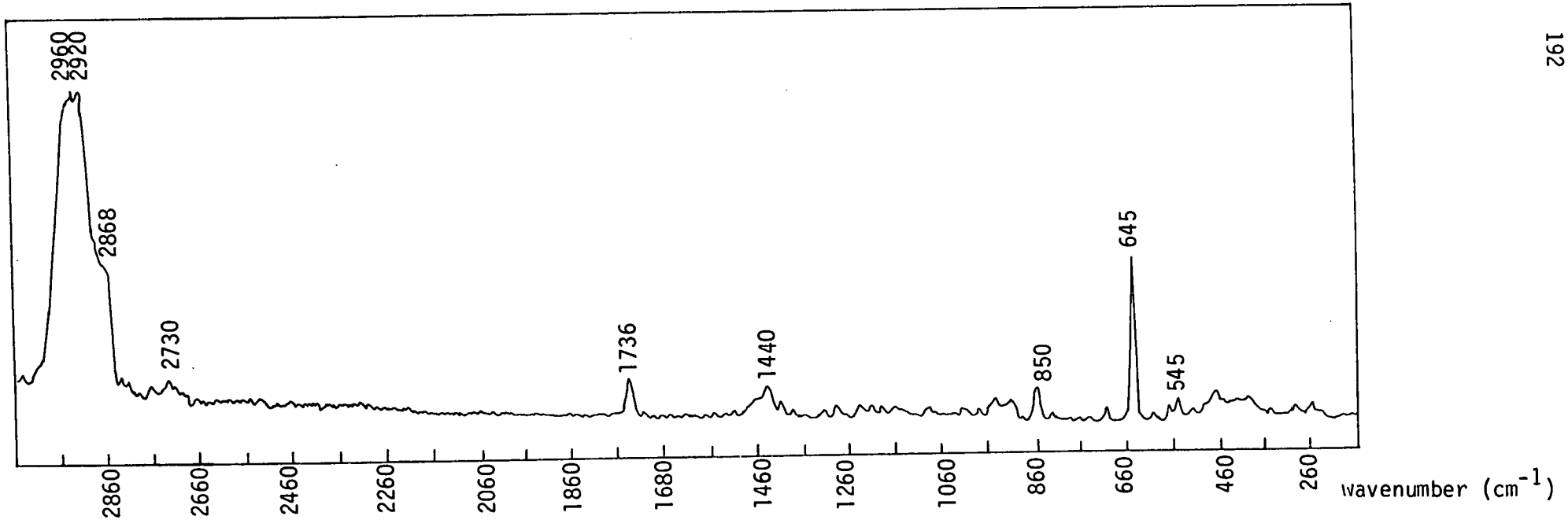
Sens. 2000 c/s
SBW 6.0 cm^{-1}
PP 10 secs.



Laser Raman Spectra of Trapped Component No. 9 from Atherosperma moschatum.

Identified as p-Cymene.

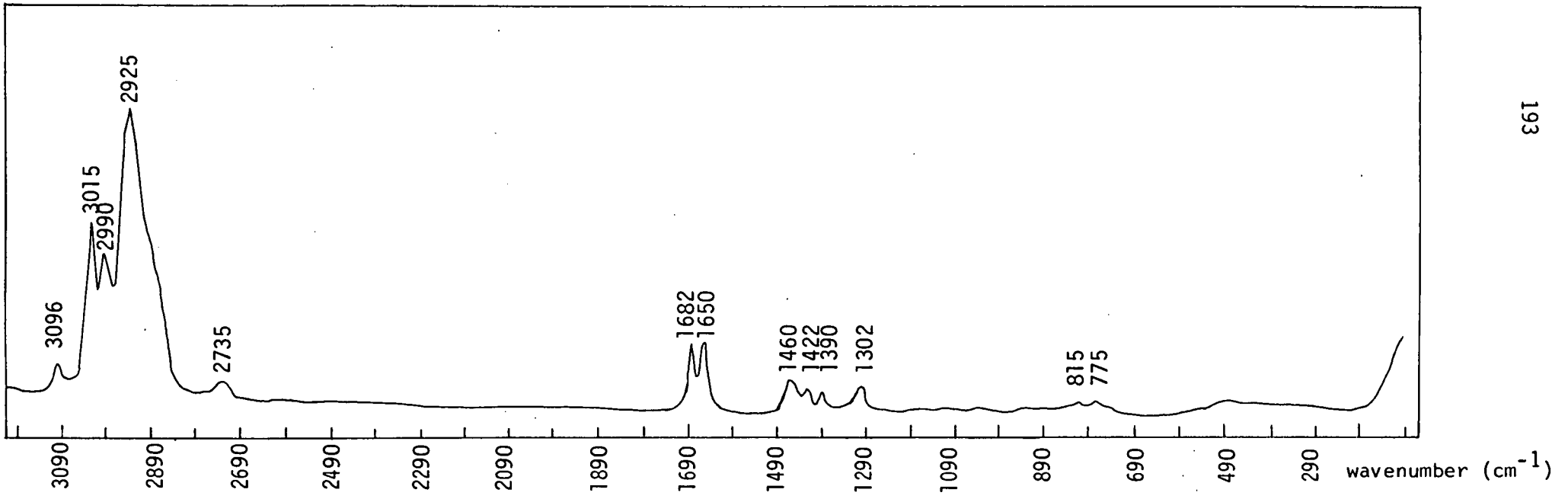
Sens. 7,500 c/s
SBW 6.0 cm^{-1}
PP 10 secs.



Laser Raman Spectra of Trapped Component No. 13 from A. moschatum.

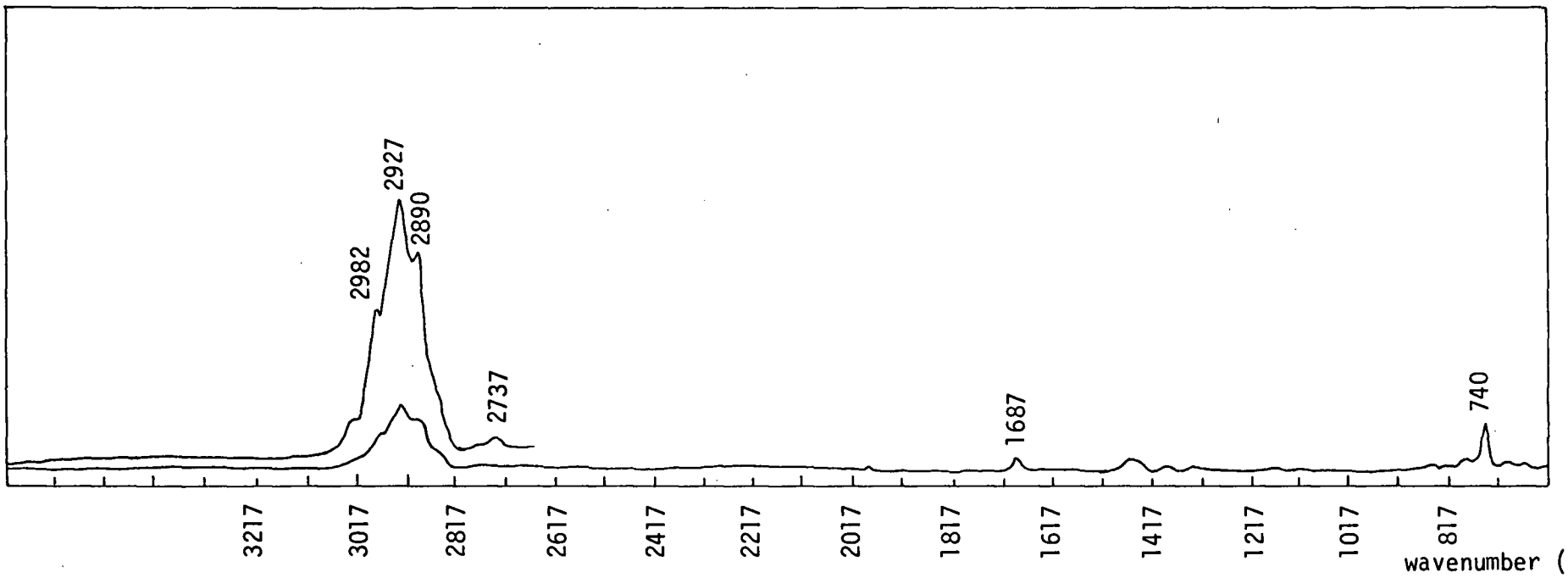
Identified as Camphor.

Sens. 3000 c/s
SBW 6 cm^{-1}
PP 10 secs.



Laser Raman Spectra of Trapped Component No. 14 from A. moschatum.
Identified as Linalool.

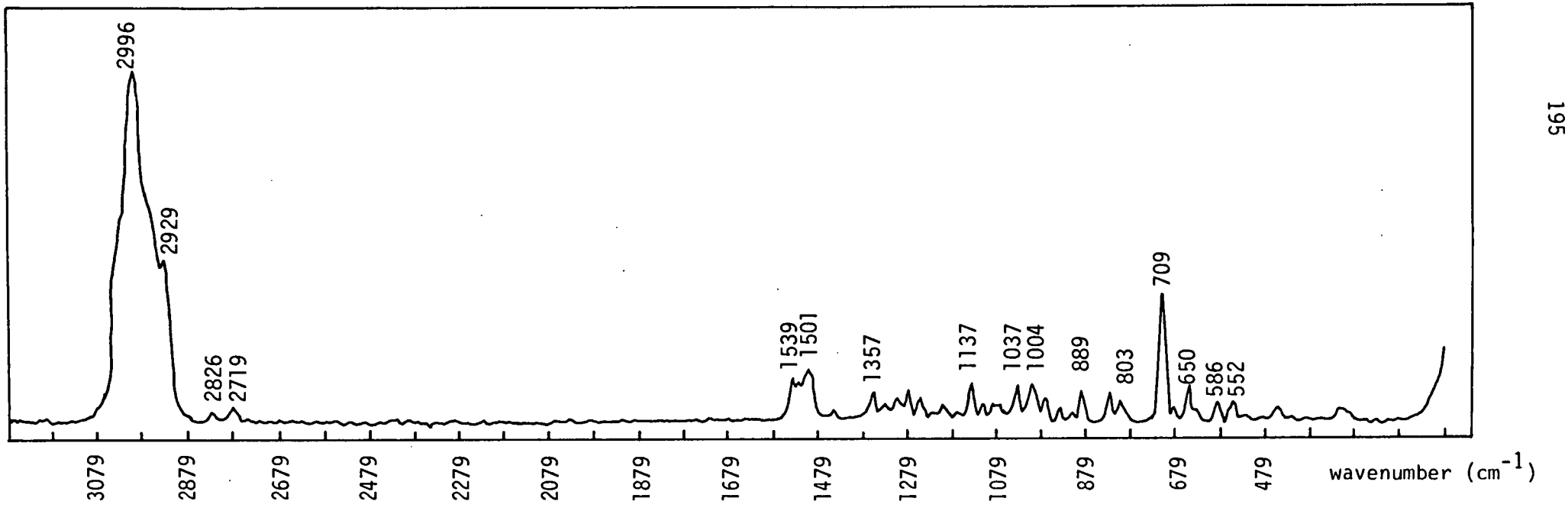
Sens. 2000 c/s
SBW 8.0 cm^{-1}
PP 10 secs.



Laser Raman Spectra of Trapped Component No. 17 from Athosperma moschatum.

Identified as Terpinen-4-ol.

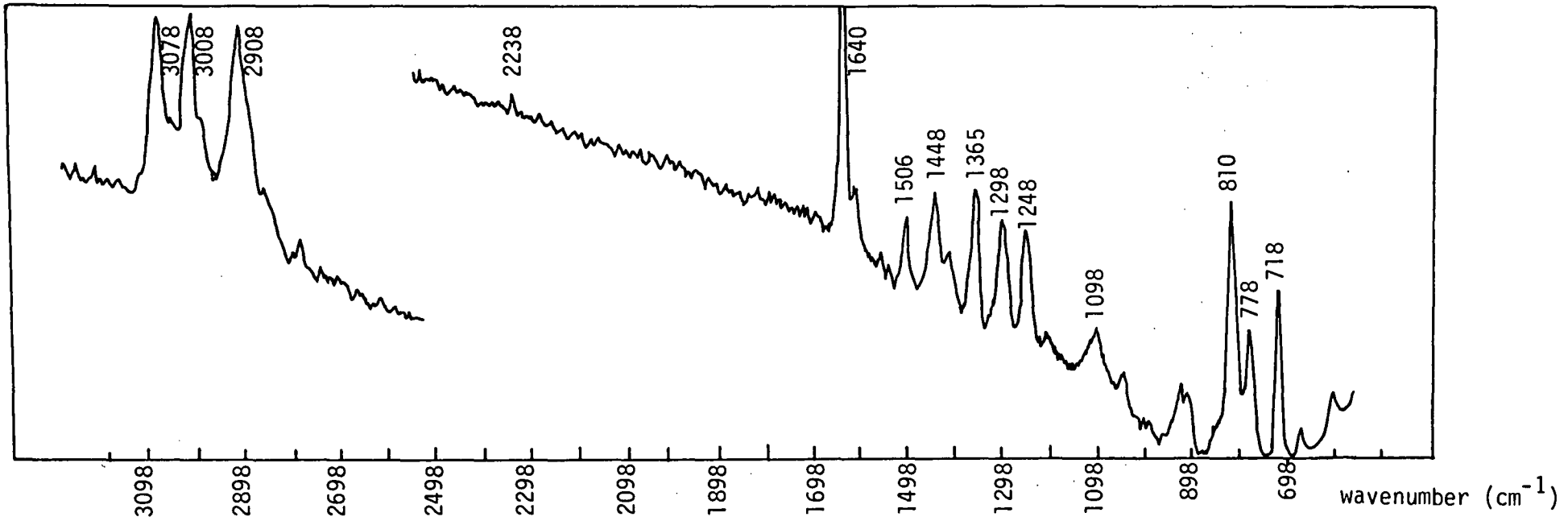
Sens. 2000 c/s
SBW 6 cm^{-1}
PP 10 secs.



Laser Raman Spectrum of Trapped Component No. 18 from Athermsperma moschatum.

Identified as Borneol.

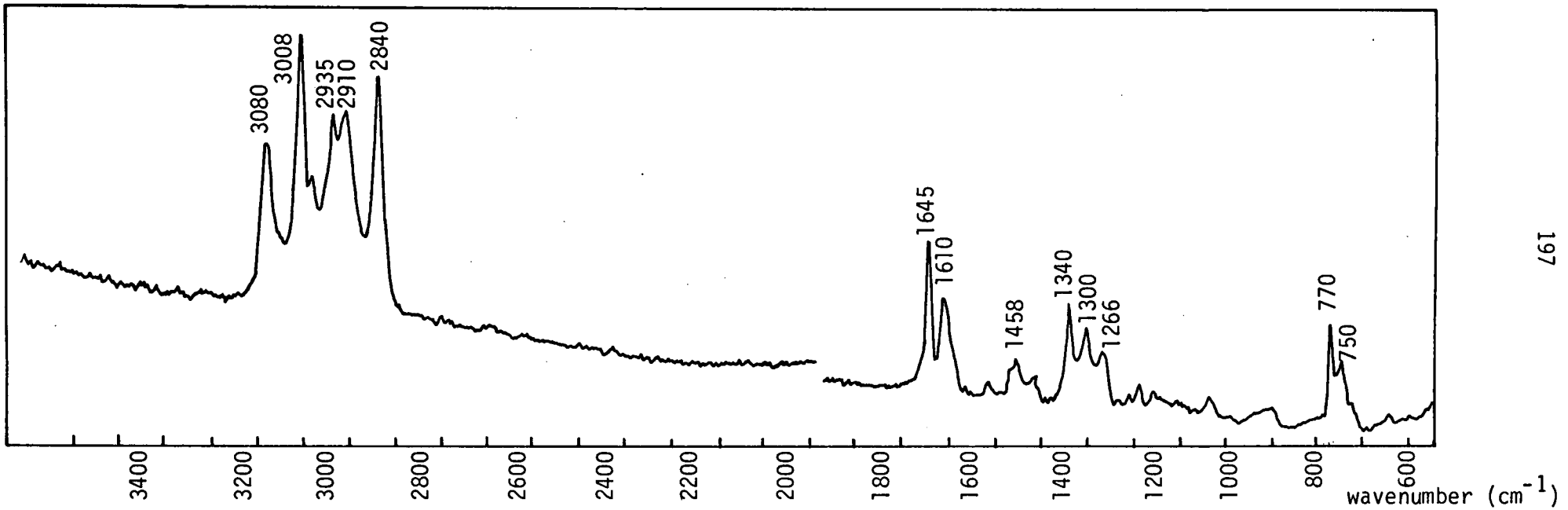
Sens. 2000 c/s
SBW 10 cm^{-1}
PP 10 secs.



Laser Raman Spectra of Trapped Component No. 22 from A. moschatum.

Identified as Safrole.

Sens. 1000 c/s
SWB 8.0 cm^{-1}
PP 20 secs.



Laser Raman Spectrum of Trapped Component No. 23 from Atherosperma moschatum.

Identified as Methyl Eugenol.

Sens. 1000 c/s
SBW 8.0 cm^{-1}
PP 10 secs.